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MALCOLM STIRLING WHITEWAY

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
MARCH 2, 1955	CANADA

Permanent Address — Résidence fixe

20 DAY AVENUE
DARTMOUTH NOVA SCOTIA
B2W 2V6

Title of Thesis — Titre de la thèse

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1983	ASAD AHMED

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SOME ASPECTS OF MITOTIC RECOMBINATION IN HAPLOID *SACCHAROMYCES CEREVISIAE*

by

MALCOLM STIRLING WHITEWAY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SOME ASPECTS OF MITOTIC RECOMBINATION IN HAPLOID SACCHAROMYCES CEREVISIAE submitted by MALCOLM STIRLING WHITEWAY in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

Asad Ahmed
Supervisor

P.C. van Berkel

A.R. Morgan

A. Kuspira

John B Bell

R.T.C.

External Examiner

Date Nov. 30, 1982

ABSTRACT

Duplications of DNA segments were produced in haploid cells of *Saccharomyces cerevisiae* by introduction of DNA through transformation. Integration of a non-replicative plasmid was used to generate direct repeats of sequences at the arginine permease (*CAN1*) locus. In a similar set of experiments transformation with an autonomously replicating plasmid was used to form extrachromosomal repeats of sequences of the endogenous yeast plasmid Scp1. These duplications were used to investigate the effect of both the duplication structure and the *RAD52* gene product on mitotic recombination involving repeated sequences.

A non-replicative plasmid containing a mutant allele of the *CAN1* gene constructed *in vitro* was introduced into cells with a wild-type allele at this locus. Integration of the plasmid at the *CAN1* locus destabilized the canavanine sensitive phenotype, while integration at other sites in the genome did not. The instability was due to intrachromosomal events involving repeated sequences. These included gene conversions between the wild-type and mutant sequences, which required the *RAD52* gene product, and resolution of non-tandem duplications, some of which were *RAD52* independent. The frequency of the events involving the duplicated segments did not depend directly on the size of the repeated sequences.

S. cerevisiae strains which contained a chromosomal can1 mutant allele and carried the CAN1 gene on an autonomously replicating chimeric plasmid were also unstable for the canavanine sensitive phenotype. This instability was due to plasmid loss and to recombination between the chimeric plasmid and the endogenous yeast plasmid Scp1. This recombination generated a plasmid containing only yeast DNA, and separated the arginine permease gene from the selectable marker of the chimeric plasmid. Formation of this novel plasmid was reduced but not eliminated in strains containing the rad52-1 mutation, and its presence was often associated with loss of Scp1.

In both plasmid and intrachromosomal recombination, similar structures can have different requirements for the RAD52 gene product. This suggests that there is a discriminatory capacity to the action of the RAD52 gene product.

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INTRODUCTION

Mitotic recombination involves the exchange of sequence information between DNA duplexes within vegetative cells. Recently, mitotic recombination has been implicated in a wide variety of processes, ranging from the generation of antibody diversity during the development of the immune system in mammals (Seidman et al., 1979) to the evolutionary maintenance of sequence homogeneity among members of repeated gene families (Szostak and Wu, 1980; Nagylaki and Petes, 1982). Recombination of DNA sequences in somatic cells (mitotic recombination) therefore has important and varied cellular functions.

Mitotic recombination was initially described in Drosophila melanogaster. Flies that were heterozygous in the trans configuration for linked cuticular markers could give rise to adjacent patches of cuticular cells that each expressed one of the recessive markers in a homozygous state. The formation of these "twin spots" was taken as evidence for somatic (mitotic) crossing over between the marker genes and their centromere (Stern, 1936). Subsequent work extended these findings to a number of lower eukaryotes, such as Aspergillus nidulans (Pontecorvo et al., 1954) and Ustilago maydis (Holliday, 1961), and to higher plants (Vig and Paddock, 1970; Christianson, 1975) and mammals (Gruneberg, 1966).

The phenomenon of mitotic recombination has been extensively studied in the yeast Saccharomyces cerevisiae (reviewed by Kuntz and Haynes, 1981; Esposito and Wagstaff, 1981). Yeast is a very suitable organism for such studies. A large number of well characterized genetic markers have been isolated in haploid yeast cells (Mortimer and Schild, 1980) and these can be combined in diploids in arrangements that allow efficient monitoring of mitotic recombination. The genetic structure of these recombinants in diploids can be accurately assessed by subsequent induction of meiosis (Roman, 1956). Recent developments in recombinant DNA technology have allowed the manipulation of well-characterized yeast DNA sequences, and the construction of haploid yeast cells in which mitotic recombination can be studied (Scherer and Davis, 1980; Szostak and Wu, 1980; Jackson and Fink, 1981).

Two kinds of homologous mitotic recombination events can be recognized genetically in S. cerevisiae. These are reciprocal (or crossing over), and non-reciprocal (or gene conversion). Reciprocal mitotic recombination was initially detected in UV-irradiated diploid cells that were heterozygous for a galactose fermentation gene. The frequent uncovering of the recessive marker was attributed to reciprocal exchange between the gene and its centromere (James, 1955; James and Lee-Whiting, 1955). Mitotic gene conversion was first observed in diploid cells that

contained homologous chromosomes each carrying different, non-complementing mutant alleles (heteroalleles) of a locus that conferred an auxotrophic requirement. In this situation, the frequent formation of prototrophic colonies was shown to result from the replacement of one allele with the wild type allele without the associated formation of a doubly mutant gene. Therefore, the event was considered to be non-reciprocal (Roman, 1956; Roman and Jacob, 1958).

Subsequent analyses have further defined the process of mitotic recombination in S. cerevisiae. The suggestion that radiation induced homozygosis of recessive markers could be due to mitotic crossing over was given strong support by the observation that linked markers could be made homozygous at the same time. When this occurred, the pattern was almost invariably that all markers centromere distal to a certain point became homozygous, while are markers centromere proximal to this point remained heterozygous. The linkage relationships established on the basis of associated homozygosis corresponded to the meiotic linkage map (Nakai and Mortimer, 1969). This provided conclusive evidence for the existence of strand exchange in mitotic cells of S. cerevisiae. Further experiments using a system in which each reciprocal product of an exchange could be phenotypically detected showed that colonies arose that were sectored for the two phenotypes (Zimmermann, 1973). These sectored colonies were equivalent to the "twin-spots" in the somatic

cells of multicellular organisms, and provided definitive evidence for the reciprocal nature of the event.

The basis for gene conversion events is less clear than that for reciprocal events, which apparently involve strand exchange. It appears that the process of conversion involves replacement of the mutant sequence of one allele with the wild type sequence from the other. This event is very precise; analysis of the resulting functional allele through the properties of its enzyme product (Zimmermann, 1968) and its restriction map (Scherer and Davis, 1980) show that the gene is indistinguishable from wild-type. Meiotic studies have shown that conversion is accurate to the level of a single base pair (Fogel and Mortimer, 1970), and similar studies have been performed for mitotic gene conversion (Roman, 1956).

Recombination Models

Although there is convincing genetic evidence for reciprocal exchanges and gene conversions during mitosis, the molecular events that occur during recombination have not been well defined. The paucity of information as to the nature of the recombination process has resulted in the development of a number of models which attempt to explain the phenomenon on the basis of plausible enzymatic and physical steps. Such models were initially developed to explain the more thoroughly studied process of meiotic

recombination, and have been subsequently modified to fit the special properties of mitotic recombination. The models that have been most successful in explaining the varied data on meiotic recombination fall into two classes - those which postulate the formation and repair of heteroduplex regions at the site of recombination, and those which invoke localized replication to permit sequence exchange without the formation or repair of extensive heteroduplex regions.

The initial formulation of the heteroduplex intermediate model invoked a concerted breakage and exchange of DNA strands of like polarity from the two chromatids involved in the recombination event (Holliday, 1964). This exchange generated a half-chromatid chiasma with symmetrical heteroduplex regions on the two recombining chromatids. Conversion was assumed to result from the repair of mismatched bases within the regions of heteroduplex. Reciprocal recombination resulted from subsequent breakage of the DNA strands of the recombining chromatids that were not exchanged initially, while breakage of the exchanged strands at the cross-connection left the flanking DNA sequences in the parental configuration.

Molecular model building has shown that the half-chromatid chiasma is structurally plausible because it involves satisfactory bond angles, and does not require any unpaired bases (Sigal and Alberts, 1972). The molecular structure has appealing attributes; the cross-strand

exchange may migrate freely in either direction due to rotary diffusion (Meselson, 1972) and isomerization can generate reciprocal recombination of the DNA sequences flanking the heteroduplex region to produce a recombinant configuration of the outside markers (Sigal and Alberts, 1972). These characteristics of the half-chromatid chiasma or Holliday structure can explain meiotic data that suggest that heteroduplex regions can extend for variable, and sometimes considerable lengths, and that reciprocal exchange and gene conversion are closely associated and appear to be part of the same process (Fogel et al., 1978).

Recently, physical evidence for apparent Holliday structures has been obtained from yeast cells. Electron microscopic studies of the endogenous yeast plasmid (Scp1) during meiosis have detected molecules that are fused within regions of homology (Bell and Byers, 1979). These fused structures, termed chi forms, have been reported previously for other eukaryotic (Benbow and Krause, 1977) and prokaryotic (Potter and Dressler, 1976; DasGupta et al., 1981) systems, and there is convincing evidence that they are recombination intermediates.

Not all the predictions of the Holliday model fit the genetic data on meiotic recombination in S. cerevisiae. The majority of intragenic meiotic recombination in yeast consists of conversion on only one chromatid, and so presumably does not involve the symmetrical formation of

heteroduplex on the two recombining chromatids (Fogel et al., 1978,) A modification of the Holliday model postulates that the initial steps in the recombination process involve the invasion of the DNA duplex of one chromatid by a single strand from an homologous chromatid (Meselson and Radding, 1975). Therefore, the initial steps are asymmetrical and form a heteroduplex on only one chromatid; later isomerizations can generate symmetrical heteroduplexes. Biochemical evidence for the uptake of a single strand into an homologous duplex DNA molecule has been obtained from an E. coli cell free system (Shibata et al., 1979). This reaction is catalyzed by the recA protein, an important enzyme in the generalized recombination system of E. coli. Therefore, the Meselson-Radding modification of the Holliday model for recombination can explain most aspects of yeast meiotic recombination, and there is strong biochemical support for many of the postulated steps.

The observation that large deletions undergo conversion in a manner similar to point mutations (Fink and Styles, 1974; Fogel et al., 1978) is not easily explained by models that involve a heteroduplex intermediate. RecA protein mediated formation of a heteroduplex is blocked by the insertion of less than 1 kb of non-homologous DNA into one of the associating DNA strands (DasGupta and Radding, 1982). Therefore it seems unlikely that rotary diffusion would be capable of generating a heteroduplex between two DNA

molecules with extensive regions of non-homology. If such a heteroduplex was formed, it would involve a mismatch of a large sequence of DNA rather than a single nucleotide pair. It might be expected that enzymatic repair systems would more readily excise the unpaired single- stranded loop, and thus the wild type sequence would be frequently converted to mutant. However, in yeast, deletion mutations show parity of conversion, that is, conversion of the deletion to the wild-type allele is as frequent as conversion of the wild-type allele to the deletion (Fink and Styles, 1974; Fogel et al., 1978). In addition, the segregation of genetically different cells from a single meiotic product containing a haploid genome, a process termed post-meiotic segregation (pms), is one of the most convincing lines of evidence for the formation of heteroduplexes during recombination, and there is no evidence for pms at loci where the heterozygosity involves extensive regions of non-homology (Fogel et al., 1978; Klar et al., 1979).

Models that do not explain gene conversion by the formation and repair of heteroduplex can more readily accommodate conversion of deletions and other extensive regions of non-homology. Such models invoke localized replication and informational transfer, and eliminate the problems associated with the heteroduplex models (Stahl, 1979). However, they are not as successful as the heteroduplex models in explaining co-ordinate post-meiotic

segregation of adjacent intragenic markers (Fogel et al., 1978), and marker related effects of recombination that are most consistent with heteroduplex mispairs invoking a repair process (Savage and Hastings, 1981).

Attempts have been made to reconcile the difficulties of conversion of deletions with the Meselson-Radding model. The absence of pms may simply result from the fact that extensive mispairing is recognized extremely efficiently by repair systems, and so heteroduplex intermediates never survive through meiosis. The problem of generating a heteroduplex can be overcome if the hybrid DNA is formed by extensive unwinding of the DNA, followed by reassociation of strands from homologous chromosomes, as originally proposed (Holliday, 1964). This, however, eliminates one of the appealing molecular attributes of the Meselson-Radding model, the formation of extensive tracts of heteroduplex by a structure that involves no unpaired bases. In addition, it is less consistent with the apparent asymmetry of the event. A model has also been proposed that DNA synthesis within the single-stranded DNA, followed by either integration or excision of the new duplex DNA, will generate parity in conversion (Radding, 1978). Because of parity and the lack of pms, this model requires that random, single-stranded loops are made duplex. At present, there is no experimental evidence which bears on the process of efficient formation of duplex DNA from single-stranded loops. Therefore,

although the Meselson-Radding model explains the majority of the data on meiotic recombination in yeast, modifications appear necessary to reconcile it with all the available data.

Although mitotic recombination has many similarities to meiotic recombination, it has characteristic differences. Some factors, such as the low frequency of mitotic recombination compared with meiotic recombination (Thornton and Johnson, 1971), and the increased relative frequency of exchanges next to the centromere during mitosis (Malone et al., 1980), may simply reflect differences in the spatial organization of the DNA molecules at the time of recombination in meiotic and mitotic cells.

Other evidence suggests that there may be differences between the meiotic and mitotic systems in the actual recombination process. Although both meiotic conversion (Fogel et al., 1978) and spontaneous mitotic conversion (Esposito, 1978; Roman, 1980) exhibit a high frequency of associated reciprocal exchange, the processes of gene conversion and reciprocal recombination appear to be separable during mitosis. Ultra-violet irradiation enhances gene conversion without increasing associated crossing-over (Hurst and Fogel, 1964; Roman and Jacob, 1958), and acridine orange can induce gene conversion but not reciprocal recombination (Fahrig, 1979). Mutations can also uncouple the two events during mitosis. An allele of rad18

specifically enhances mitotic gene conversion but does not affect reciprocal exchange (Boram and Roman, 1976), while the rad52-1 mutation reduces mitotic recombination by eliminating gene conversion, and has no effect on reciprocal recombination (Jackson and Fink, 1981). The rad52-1 mutation appears to abolish all meiotic recombination (Prakash et al., 1980; Game et al., 1980).

A further significant difference between mitotic and meiotic recombination is in the timing of the event. Meiotic recombination occurs at the post-replicative, G2 or 4-stranded stage, while convincing evidence exists that some mitotic recombination events can be initiated (Esposito, 1978; Wildenberg, 1970) and even completed (Fabre, 1978) in the prereplicative, G1 or 2-stranded stage. However, the occurance of unequal sister chromatid exchange shows that some mitotic recombination must take place at the 4-stranded stage (Jackson and Fink, 1981).

Mitotic recombination has similarities to meiotic recombination, but has special characteristics not found in meiotic exchange, facts which are reflected in recent models of mitotic recombination. The observation that mitotic recombination events can be initiated in G1, and the frequent association between gene conversion and reciprocal exchange led to the proposal that mitotic recombination results from the formation, in G1, of Holliday structures that often involve symmetrical heteroduplexes, and that

nucleolytic cleavage, or replication through the half-chromatid chiasma producing reciprocally recombined centromere distal markers, can resolve the exchanges (Esposito, 1978). However, although a high proportion of the events which appear to involve symmetrical heteroduplex formation also involve a reciprocal exchange, a large proportion of the conversion events are asymmetrical and not highly associated with crossing over (Roman, 1980). This observation suggested that there may be two processes, one involving symmetrical heteroduplex and crossing-over, and a second, involving heteroduplex formation on only one strand, which results from unidirectional transfer of a single strand without the formation of a cross-strand exchange. This second process was termed interchromosomal transformation (Roman, 1980). Mating type interconversion apparently results from such a unidirectional conversion event (Haber et al., 1980).

Recent models for mitotic recombination in yeast involve modifications of the Meselson and Radding model which accommodate the timing of the mitotic events and the apparent separability of conversion and crossing-over during mitotic recombination.

Recombination Mutants

One way of defining the validity of these models, and of establishing the sequence of events during the mitotic recombination process, is to identify mutants that modify specific steps in the process. Two approaches have been taken to identify such mutants in yeast - direct isolation of recombination mutants, and analysis of the recombination processes in mutants that modify other aspects of DNA metabolism.

The identification of mutations directly affecting mitotic recombination has been attempted in S. cerevisiae, but with limited success. Until recently it has been possible only to monitor recombination between different alleles of genes on homologous chromosomes. However, because diploid cells would prevent the detection of recessive mutations, it was necessary to attempt to isolate mutants in disomic strains which were heteroallelic for a marker on the duplicated chromosome (Maloney and Fogel, 1980; Rodarte-Ramon and Mortimer, 1972). In addition, because spontaneous mitotic recombination is infrequent (Thornton and Johnson, 1971), it was not feasible to select directly for mutants defective in spontaneous heteroallelic recombination. Therefore, mutants were isolated which were either defective in radiation-induced recombination (Rodarte-Ramon and Mortimer, 1972) or showed enhanced spontaneous recombination levels (Maloney and Fogel, 1980). Although both studies

identified mutants, neither was successful in defining a specific biochemical defect in recombination.

The alternative approach to obtaining mutations which affect recombination is to examine the recombinational proficiency of mutants defective in DNA repair. In E. coli, several of the recombination mutants have been found to be sensitive to UV-irradiation and chemical mutagens (Clarke, 1973). Several of the S. cerevisiae strains identified during attempts to isolate recombination mutants were also sensitive to DNA damaging agents (Maloney and Fogel, 1980; Rodarte-Ramon and Mortimer, 1972). Mutants with enhanced sensitivities to DNA damaging agents, or with increased mutation rates, can be identified in haploid cells, and are therefore easier to obtain than are mutations which directly affect recombination.

Two mutations with defined biochemical defects, originally isolated on the basis of a temperature sensitive block in the cell division cycle (cdc) were found to affect mitotic recombination. Both cdc9, which codes for DNA ligase (Johnston and Nasmyth, 1978), and cdc21, which codes for thymidylate synthetase (Game, 1976), have enhanced levels of spontaneous mitotic recombination (Game et al., 1979; Kuntz et al., 1980). These different mutations enhance recombination because they increase the frequency of nicks in DNA, either through failure to efficiently ligate replication intermediates, or through increased

incorporation of, and subsequent repair of, uracil in the DNA. This common phenotype for different mutants gives support to the idea that nicking of DNA is an important, possibly rate limiting step, in the mitotic recombination process.

Many of the mutants that are sensitive to the effects of ionizing radiation (rad) have pleiotropic recombinational deficiencies. The rad50 series of mutants are generally defective in both meiotic and mitotic recombination. The rad52-1 allele (Resnick, 1969; Game and Mortimer, 1974) has been the most extensively studied of these mutants. This mutant was originally isolated on the basis of its sensitivity to X-rays (Resnick, 1969) and was later found highly sensitive to methyl methane sulphonate (Prakash and Prakash, 1977) and weakly sensitive to UV-irradiation (Lawrence and Christensen, 1976). In addition, strains carrying the rad52-1 allele have increased spontaneous mutation rates (von Borstel et al., 1971; Prakash et al., 1980), and are defective in the repair of double strand breaks in the DNA (Resnick, 1975).

The rad52-1 allele has dramatic effects on recombination. This mutation eliminates meiotic recombination (Prakash et al., 1980; Game et al., 1980) and reduces spontaneous and radiation induced mitotic recombination (Prakash et al., 1980). The decrease in mitotic recombination is apparently due specifically to a

reduction in gene conversion; the rad52-1 allele affects intrachromosomal gene conversion, but not reciprocal exchange (Jackson and Fink, 1981). Other experiments support the suggestion that the rad52-1 allele primarily eliminates gene conversions. Mating type switching, which involves a unidirectional gene conversion (Haber et al., 1980) is eliminated by the rad52-1 mutation (Malone and Esposito, 1980). Unequal sister chromatid exchange (Zamb and Petes, 1981) and chromosomal integration of circular non-replicative plasmids during yeast transformation (Orr-Weaver et al., 1981) are both reciprocal events and are not influenced by the rad52-1 mutation. However, other events whose products are reciprocal do require the rad52 gene product. Chromosomal integration of linear and gapped linear plasmids (Orr-Weaver et al., 1981) and UV-induced sister chromatid exchange (Prakash and Taillon-Miller, 1981) are both eliminated by the rad52-1 allele. The variety of events that require or do not require the rad52 gene product has led to the suggestion that the rad52 gene product is involved in repair synthesis of DNA (Orr-Weaver et al., 1981). The process of DNA synthesis is required for the asymmetrical phase of the Meselson-Radding model for generalized recombination,(and thus gene conversion), but it is not required for the reciprocal exchange. At present, the possible biochemical basis for the rad52-1 mutation is predicted from this model of recombination. If this prediction is found to be correct, it will provide strong

support for the Meselson-Radding model.

Recombination Involving Duplicated Sequences

Many of the recent advances in the understanding of genetic recombination in yeast have been achieved through the use of recombinant DNA techniques. The process of transformation of yeast with purified DNA sequences (Hinnen et al., 1978) has been used to directly analyze the recombination process (Orr-Weaver et al., 1981) and to generate chromosomal (Jackson and Fink, 1981; Klein and Petes, 1981; Scherer and Davis, 1980; Szostak and Wu, 1980) and extrachromosomal (Beggs, 1978; Broach et al., 1982) sequence duplications that can be used for the analysis of meiotic and mitotic recombination. These duplications have facilitated the study of mitotic recombination in haploid cells of S. cerevisiae, a process that was previously less amenable to study.

Recombination between naturally occurring sequence duplications has important functions in haploid yeast cells. Mating type switching (Hicks et al., 1979) and recombination between the inverted repeats of Scp1 (Beggs, 1978) are examples of highly efficient site specific recombination systems that require specialized gene products - HO for mating type switching (Strathern et al., 1979) and FLP for inverted repeat recombination (Broach et al., 1982). The recombinational switching of the genetic information at the

mating type locus controls the expression of a number of genes involved in the mating response of S. cerevisiae (Herskowitz and Oshima, 1981), and thus mitotic recombination indirectly plays a critical role in determining many aspects of the cell's physiology.

Similar recombination systems have been identified in prokaryotic cells (Bukhari and Ambrosio, 1978; Hoess et al., 1982; Zeig et al., 1977). In higher eukaryotes, site specific recombination in somatic cells has been implicated in the development of the immune system (Seidman et al., 1979; Van Ness et al., 1982), but no specialized recombination enzyme has yet been identified.

Generalized mitotic recombination between repeated sequences may be important over evolutionary time. Gene conversion has been detected between similar, non-allelic genes which code for the cytochrome c's of yeast (Ernst et al., 1981), and evidence for similar events have been detected in higher eukaryotes (Liebhaber et al., 1981). This process may increase variability by generating new alleles (Ernst et al., 1981) or reduce variability by maintaining sequence identity among repeated gene families (Nagylaki and Petes, 1982). Reciprocal recombination between tandemly repeated genes may also serve to maintain sequence homogeneity (Szostak and Wu, 1980); reciprocal recombination between dispersed genetic elements can generate chromosomal rearrangements (Roeder and Fink, 1980). Thus generalized

mitotic recombination probably plays an important role in determining the genetic structure of S. cerevisiae.

Because processes such as mating type switching and Scp1 inverted repeat recombination require specialized gene products, and because recombination between dispersed or tandemly repeated DNA sequences may be hard to detect phenotypically, generalized mitotic recombination in haploid cells has been studied chiefly through artificially created duplications (Jackson and Fink, 1981; Scherer and Davis, 1980) or through insertion of genetic markers into naturally occurring repeated sequences (Szostak and Wu, 1980). These approaches have increased the ability to monitor mitotic recombination. Introduction of genetically detectable markers into the ribosomal genes has allowed for the detection and analysis of unequal sister chromatid exchange, a process that could not previously have been observed (Szostak and Wu, 1980).

Objective of the Present Study

Although genetic studies have suggested that the structure of the recombining sequences plays a role in the recombination process, this has not been extensively studied at a molecular level. Introduction of physically defined DNA sequences into yeast cells by transformation can generate a variety of recombinational substrates. These can be used to monitor the effect of the structure of the recombining

sequences on generalized mitotic recombination in haploid yeast cells.

In the present study duplications of DNA from the CAN1 locus of S. cerevisiae have been used to study generalized mitotic recombination in haploid yeast cells. The CAN1 locus encodes the structural gene for arginine permease (Grenson et al., 1966); strains which carry the wild-type allele are sensitive to the arginine analogue canavanine, while strains with the mutant allele at the CAN1 locus are resistant to the toxic effects of this compound (Fink, 1970). Therefore, in haploid strains which carry both mutant and wild-type alleles of the CAN1 locus, canavanine resistance provides a positive selection for the product of a recombination event which removes or inactivates the wild type sequence. This system allows for flexibility in the range of structures in which recombination can be assessed, because recombinational processes that do not directly involve the CAN1 sequence, but instead involve adjacent DNA sequences, can still lead to loss of the CAN1 function and thus can be detected.

The present study will investigate the recombinational behaviour of a variety of structures. These include duplicated chromosome sequences that differ in size, sequence, and in the distance between the duplications, as well as duplicated extrachromosomal sequences on autonomously replicating plasmids. In addition, it will study the role of the RAD52 gene product in the

recombination of these structures, and will therefore assess the interactions between DNA structures and enzymatic functions during generalized mitotic recombination. This will allow for a more detailed understanding of the function of DNA structures in the recombination process.

MATERIALS AND METHODS

GROWTH MEDIA

E. coli

Complete (LT) - The complete medium was Luria broth plus thymine. This contained per liter: 12.0 g Bacto-tryptone; 10.0 g sodium chloride; 5.0 g Bacto-yeast extract; 1.0 g dextrose; and 50 mg thymine. Solid complete medium was LT containing 12.0 g/l Bacto-agar. Antibiotic medium was solid complete medium with 12.5 mg/l tetracycline and/or 100 mg/l ampicillin. The antibiotics were added after the medium was autoclaved.

Minimal - The minimal medium was Davis minimal plus thiamine. This contained per litre: 7.0 g dipotassium phosphate; 2.0 g monopotassium phosphate; 2.0 g dextrose; 1.0 g ammonium sulphate; 0.5 g sodium citrate; 100 mg magnesium sulphate, and 10 mg thiamine hydrochloride. Required amino acids were added at 20 mg/l. Solid medium contained 12.0 g/l Bacto-agar, and

antibiotics were added as for complete medium.

S. cerevisiae

Complete (YEPD) - The complete medium was yeast extract, peptone, dextrose. This contained per litre: 20.0 g Bacto-peptone; 10.0 g Bacto-yeast extract, and 10.0 g dextrose. Solid complete medium was YEPD containing 20.0 g/l Bacto-agar.

Minimal (SC) - The supplemented minimal medium was synthetic complete. This contained per litre: 20.0 g dextrose, 6.7 g Bacto-yeast nitrogen base without amino acids, and supplements of 40 mg leucine, 40 mg lysine hydrochloride, 30 mg adenine hydrochloride, 30 mg tryptophan, 20 mg histidine hydrochloride, 20 mg methionine, and 20 mg uracil. Solid medium contained 20.0 g/l Bacto-agar.

Omission - Omission medium was solid or liquid SC missing one or more supplements, and is described in the text as "SC-missing

supplement(s).

Canavanine - Canavanine medium was solid SC or omission medium containing 60 mg/l canavanine sulphate, and is described in the text as "SC+canavanine".

Regeneration - Regeneration medium contained per litre: 182.2 g sorbitol, 30.0 g Bacto-agar, 20.0 g dextrose, 6.7 g Bacto yeast nitrogen base without amino acids, and 4 times the supplements as for SC-leucine.

Sporulation (FS) - Sporulation medium contained per litre: 15.0 g Bacto-agar, 9.8 g potassium acetate, 2.5 g Bacto yeast extract, 1.0 g dextrose, and supplements as for SC.

STRAINS

The E.coli and S.cerevisiae strains used in this study are listed in Table 1.

YEAST METHODS

Diploid yeast strains with specific genetic markers were constructed, using standard procedures, by making crosses between haploid strains that carried the required mutations. Haploid strains with specific genetic markers were generated by sporulation of diploid cells containing the desired mutations (Sherman et al., 1981). Matings were performed by mixing together freshly grown haploid cells of opposite mating types. Diploids were selected either by direct micromanipulation of zygotes after 6 hours mating at 30°C, or by selecting, by cross-print matings, cells prototrophic for complementing auxotrophies in the haploids.

Diploids were sporulated on FS medium for 3 to 10 days at 23°C. Asci were suspended in a 10% solution of Glusulase and digested at room temperature for 30 min. The digested asci were streaked on plates and dissected directly on the agar surface with a de Fonbrune micromanipulator. The spores were allowed to grow at 30 or 35°C for 2 days and then were tested for genetic markers. Auxotrophic markers were scored

TABLE 1

E. coli		Markers	Source
delta 303-2 recA		deletion (gal-chl-pgl) thr⁻leu⁻ lac⁻ thi⁻ sup⁺ recA⁻ SmR	Dr. A. Ahmed
JF1754		hsdR lac⁻ gal⁻ metB hisB leuB	Dr. D. Bendiak

S. cerevisiae	Mating Type	Markers	Source
MSW5-21A	alpha	leu2-3,2-112; his3-11,3-15; trp1-1	Present study
MSW6-4A	alpha	arg4-17; rad52-1	"
MSW28-10C	alpha	leu2-3,2-112; his3-11,3-15; trp5-2; can1-100; ura3-1	"
MSW72-10C	a	leu2-3,2-112; his1-7; ade⁻; trp⁻; arg4-17; rad52-1	"
MSW85-15B	a	leu2-3,2-112; his1-7; can1-100; ura3-1; ade2-1	"
MSW152-1A	a	leu2-3,2-112; his1-7; can1-100; ura3-1; rad52-1	"
GRF18	alpha	leu2-3,2-112; his3-11,3-15; can1	Dr. G.R. Fink
SS101	a	pet⁻	Dr. D. Livingston
X2180-1A	a	gal2; mal⁻	Stock Centre
YO555-19A	a	leu2-3;2-112; his1-7; trp1-1	Dr. S.K. Quah
YT6-2-1L	alpha	leu2-3,2-112; his4-419; can1 (cir°)	Dr. V. MacKay

S. cerevisiae Transformants

MSW75-2C	alpha	leu2-3,2-112; his⁻; trp⁻; arg4-17; rad52-1 but LEU2 from p47-3 at *CAN1* locus	Present study
MSW5-21A/p47-3#1-5	alpha	same as MSW5-21A but LEU2 from p47-3	"
MSW28-10C[TLC-1]	alpha	same as MSW28-10C but LEU2 CAN1 from TLC-1	"
MSW152-1A[TLC-1]	a	same as MSW152-1A but LEU2 CAN1 from TLC-1	"
YT6-2-1L[TLC-1]	alpha	same as YT6-2-1L but LEU2 CAN1 from TLC-1	"

by replica plating to omission media. Radiation sensitivity was tested by replica plating to YEPD plates and irradiating with 40 krad of gamma rays from a Gammacell 200 ^{60}Co source (Atomic Energy of Canada Ltd.). Canavanine sensitivity was established by replica plating to SC medium supplemented with 60 micrograms/ml canavanine sulphate. Mating type was determined by crossing the haploid strains to testers of defined mating type and scoring mating either by visual inspection of the mating mixture or by the formation of cells prototrophic for complementing auxotrophies in the haploids.

TRANSFORMATION

E. coli Transformation

E. coli cells were made competent for transformation by treatment with calcium chloride (Cohen et al., 1972). An overnight culture of bacteria was diluted 1 to 40 in LT broth and shaken at 30°C for 1.5 hr. The cells were chilled on ice, harvested by centrifugation at 3,000 x g for 5 min., and suspended in 0.5 volume of ice cold 50 mM calcium dichloride. The cells were again collected by centrifugation, suspended in 0.1 volume ice cold 50 mM calcium dichloride containing 15% glycerol, and distributed

in 0.3 ml aliquots to sterile 1.5 ml microfuge tubes. These competent cells were stored at -70°C until use.

Transformation was induced by heat shock (Cohen et al., 1972). Competent cells were thawed on ice, and 0.2 ml mixed with 0.1 ml of a DNA solution adjusted to 50 mM calcium dichloride. The mixture was held on ice for 45 min., heated to 42°C for 2 min., cooled on ice for 10 min., and diluted to 1.0 ml with LT broth. The cultures were shaken at 30°C for 2 hr. to allow expression of drug resistance markers, and then suitable dilutions were spread on LT+amp or LT+tet plates. The plates were incubated at 37°C overnight.

Yeast transformation

Yeast spheroplasts were transformed by calcium chloride and polyethylene glycol treatment (Hinnen et al., 1978) as modified by V. MacKay (personal communication). A saturated culture of yeast cells was diluted 1 to 30 in YEPD and grown for 5 hr. at 30°C. The cells were harvested by centrifugation at 3,000 x g for 5 min and suspended in 0.3 volume of 1M sorbitol, 0.05M disodium EDTA pH8.0 containing 0.35% Beta-mercaptoethanol. The cells were incubated at room temperature for 20 min. and collected by centrifugation. The cells were suspended in 0.1 volume 1M sorbitol, 0.1M Na citrate pH5.4 containing 1% Glusulase and incubated at 30°C. Spheroplast formation was monitored by testing 0.1 ml aliquots for clearing when adjusted to 0.5 ml 1M sorbitol,

2% SDS. When spheroplasting was complete, the spheroplasts were collected by centrifugation, washed in 0.1 volume CaS (1M sorbitol, 0.05M calcium dichloride) and suspended in 0.01 volume CaS. The spheroplasts were mixed with 10 micrograms DNA, incubated at room temperature for 15 min., diluted with 10 volumes Tris pH7.5 containing 20% PEG 4,000, and incubated a further 20 min. at room temperature. The transformed spheroplasts were collected by centrifugation and suspended in 0.03 volume of 1M sorbitol, 33 mM calcium dichloride and 33% YEPD, and gently shaken at 30°C for 30 min. Suitable dilutions of the spheroplasts were mixed with 7.0 ml molten regeneration agar held at 50°C, and immediately poured on SC-leucine plates. These plates were incubated at 30°C for 5 days.

AGAROSE GEL ELECTROPHORESIS

Restriction enzyme digests and rapid plasmid preparations were analyzed by agarose gel electrophoresis (Cohen et al., 1973; McDonnell et al., 1977). A solution of 1% agarose (Sigma type II, medium EEO) in TEA (0.05M Tris, 0.02M disodium acetate, 0.002M disodium EDTA, 0.018M NaCl, pH8.05) was melted and cast in a horizontal slab gel electrophoresis apparatus (Aquebogue Machine and Repair Shop, Aqueboque N.Y. or Bethesda Research Laboratories Inc., Rockville MD.). Analytical gels were cast with 13 or 14 slots; preparative gels with a single large slot. DNA samples adjusted with glycerol, SDS and BPB, as described under restriction analysis, were applied in 20 microlitre aliquots and run in TEA pH8.05 until the BPB tracking dye reached the end of the gel.

The gels were stained in a solution of 0.5 microgram/ml ethidium bromide for 30 min. and visualized with a long wave UV light transilluminator (Ultra-violet Products Inc., San Gabriel, CA) Photographs were taken with a Polaroid MP4 camera using Polaroid Type 57 film and a Kodax Wratten #9 gelatin filter.

SOUTHERN ANALYSIS

Nick translation

Radioactivity was incorporated into purified DNA by incubation of nicked DNA with DNA polymerase I and ^{32}P labled nucleotides (Davis et al., 1980). Reactions contained (in a 25 microlitre volume): 0.05M Tris pH7.5; 0.01M Mg sulphate; 0.001M DTT; 50 microgram/ml BSA; 0.02 mM dATP, dGTP and TTP; 10 microlitres ^{32}P dCTP in Tricine (3000 Ci/mMole, New England Nuclear, Boston, Mass.), 5 units of DNA polymerase I 0.125 ng DNase I, and 1 microgram DNA. Reactions were incubated at 14°C for 3 hours, and stopped by the addition of 25 microlitre of a solution containing 0.02M trisodium EDTA, 2 mg/ml sonicated calf thymus DNA, and 0.2% SDS. The stopped reaction was passed over a Sephadex G-50 column equilibrated with 10 mM Tris, 1 mM trisodium EDTA pH7.5. The initial peak of radioactivity was collected in 0.5 ml fractions, and the amount of ^{32}P incorporation was estimated by diluting 1 microlitre of each fraction in 3.0 ml Aquasol 2 (New England Nuclear, Boston, Mass.) and counting in a Beckman LS 7500 liquid scintillation counter.

Southern blotting

DNA fragments were transferred from agarose to nitrocellulose by blotting with a high salt buffer (Southern, 1975). The DNA was fractionated on a 1% agarose gel, and the gel was treated with 0.25M HC1 for 15 min. at room temperature to hydrolyze large DNA fragments and thus ensure uniform transfer (Wahl et al., 1979). The DNA in the gel was denatured at room temperature by a 40 min. immersion in 0.5M NaOH, 1.5M NaCl; then neutralized at room temperature by a 40 min. immersion in 1.0M Tris, 3M NaCl pH7.0 (Davis et al., 1980). The gel was then placed on a wick of Whatman 3 MM paper, saturated in 1.0M Tris, 3M NaCl pH7.0, that extended into a solution of 20 x SSPE (20 mM disodium EDTA, 0.2M Na diphosphate 0.16M NaOH, 3.6M NaCl pH7.0) (Davis et al., 1980). The gel was overlaid with a sheet of nitrocellulose (Schleicher and Schuell, Keene, New Hampshire) that had been soaked in distilled water and then immersed in 20 x SSPE. The nitrocellulose was then overlaid, first with 5 sheets of Whatman 3 MM paper saturated with 20 x SSPE, and then with a weighted stack of paper towels. The gel was blotted for 3 hours, then the nitrocellulose sheet was washed in 2 x SSPE for 10 min. and baked for 2 hours in a vacuum oven at 80°C.

Hybridization

Nick translated probes were hybridized to DNA fragments immobilized on nitrocellulose (Davis et al., 1980). The nitrocellulose filter was incubated at 42°C for 24 hours in a solution containing 50% formamide, 5 x SSPE pH7.0, 0.3% SDS, 100 microgram/ml sonicated calf thymus DNA, and 1 to 5 x 10^4 cpm of heat denatured ^{32}P labeled probe. The filter was washed at 45°C with 3 changes of 100 ml of 2 x SSPE pH7.0 containing 0.6% SDS, then it was dried and covered with plastic wrap prior to autoradiography.

Autoradiography

Hybridized nitrocellulose filters were exposed to Kodak XRP-1 X-ray film in X-ray cassettes. The cassettes were stored at -70°C. After exposure the films were developed with Kodak X-ray film developer and fixed in Kodak Rapid Fixer following the manufacturer's instructions.

ENZYMATIC TREATMENT OF DNA

Restriction Analysis

DNA samples were analyzed by sequence specific restriction enzyme cleavage (Cohen et al., 1973). Analytical restrictions of bacterial plasmids were performed in 250 microlitre microfuge tubes. Reactions contained, in a 50 microlitre total volume - 10 microlitre of a reaction mixture specific to the restriction enzyme following the supplier's instructions, approximately 2 micrograms of purified plasmid DNA in 40 microlitres TE pH7.2, (10mM Tris, 1mM disodium EDTA) and 3 units of enzyme. Restrictions were incubated at 37°C for 2 to 4 hours and stopped either by the addition of 20 microlitres of dye solution (25% glycerol, 5% SDS, 0.025% BPB) or by heating to 70°C for 10 min. Incubation times required to partially restrict samples were established empirically for each reaction.

Restrictions used for Southern blotting contained approximately 15 microgram of bulk yeast DNA in a 50 microlitre reaction. These digestions were stopped by the addition of 20 microlitres of dye solution. Aliquots of 20 microlitres of the dye-stopped bulk yeast or bacterial plasmid digests were analyzed by agarose gel electrophoresis.

Reactions were proportionally scaled up to a 250

microlitre reaction volume for preparative restrictions. These were performed in 1.5 ml microfuge tubes and stopped by the addition of 100 microlitres of dye solution. The entire 350 microlitre volume was used for preparative agarose gel electrophoresis.

Alkaline phosphatase treatment

Restricted plasmid DNA was treated with calf intestinal alkaline phosphatase (CIAP) to prevent vector recircularization (Goodman and MacDonald, 1979). The restricted DNA was adjusted with 0.01 volume of 1M Tris pH9.0 and incubated with 1.5 units CIAP for 30 min. at 65°C. This preparation was treated with a further 1.5 units CIAP at 65°C for 30 min, then extracted six times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and dialized against TE pH7.2.

Ligations

DNA fragments generated by restriction enzyme digestion were covalently joined with T4 ligase and ATP (Bolivar et al., 1977a). Reactions contained, in a 50 microlitre reaction volume - 10 microlitre of 0.33M Tris pH7.6, 0.033M MgCl, 0.05M DTT, 0.0025 M ATP; 40 microlitre the DNA fragments being ligated; and 5 units T4 ligase. Reactions were incubated at 12°C for 16 hours.

DNA PURIFICATION

Yeast DNA isolation

Bulk yeast DNA was isolated by chloroform/isoamyl alcohol extractions of SDS lysed spheroplasts (Cryer et al., 1975). Strains containing only stable chromosomal markers were grown to late logarithmic phase in 100 ml liquid YEPD; strains carrying unstable plasmid markers were grown to late logarithmic phase in 250 ml omission medium suitable to maintain the plasmid. Harvested cells were treated with beta-mercaptoethanol and Zymolyase 5000 and lysed in 10 ml of 1 M sorbitol, 0.1M disodium EDTA pH7.5 adjusted to 1% SDS and 0.05 mg/ml Proteinase K. After lysis, chloroform/isoamyl alcohol extraction, and ethanol precipitation of the nucleic acids, the DNA and RNA were dissolved in 3.0 ml of 0.15M NaCl 0.015 M Na citrate at pH7.0. This solution was digested with 0.1 ml of a 1 mg/ml preparation of RNase A in 0.05M Na acetate pH5.0, that had been heated to 90°C for 10 minutes (Myers et al., 1976); the digestion was for 1 hour at 37°C. The digested solution was extracted with an equal volume of chloroform/isoamyl alcohol. Two volumes of 95% ethanol were added to the aqueous phase, and the DNA was spooled on a glass rod, dried, resuspended in TE pH7.2 at a concentration of 1 mg/ml, and stored at -20°C.

Yeast plasmid DNA isolation

Plasmid DNA from yeast cells was isolated by acidified phenol (Zasloff et al., 1978). Purified bulk DNA in TE pH7.2 was extracted once with an equal volume of phenol saturated with 0.05M Na acetate pH4.0, and the aqueous phase neutralized with 0.05 volume of 1M Tris pH8.6. The neutralized solution was dialyzed against TE pH7.2 and stored at -20°C.

Bacterial plasmid DNA isolation

Bacterial plasmid DNA was purified on a cesium chloride/ ethidium bromide density gradient (Thompson et al., 1974). A saturated 500 ml culture was harvested and lysed as described, with the lysozyme treatment increased to 5 minutes. The cleared lysate was layered on a 3.0 ml CsCl cushion (61.7% w/w CsCl in water) and spun at 70,000 x g (23,000 rpm) in a Beckman SW27 rotor for 20 hours. After centrifugation the lower 7.0 ml of the tube was collected and adjusted with 5.2 ml of 61.7% CsCl and 0.5 ml of a 10 mg/ml ethidium bromide solution. This mixture was divided into two 6.0 ml volumes and spun at 122,000 x g (43,000 rpm) for 24 hours in Beckman polycarbonate tubes in a Beckman Ti-50 rotor. After centrifugation the tubes were illuminated with long wave UV light, and the lower fluorescing band containing the plasmid DNA was removed. The plasmid DNA solution was extracted 3 times with n-butanol saturated with

cesium chloride, and dialyzed against 2 changes of TE pH7.2. The DNA concentration was determined spectrophotometrically, and the sample stored at -20°C.

Electroelution

Specific restriction fragments were isolated by electroelution from agarose gels. Preparative restrictions were run on 1% agarose gels, stained with ethidium bromide, and visualized by long wave UV light. The band of interest was cut from the gel and placed in a dialysis bag containing 0.5 ml TEA pH8.05. The DNA was eluted by electrophoresis in TEA pH8.05 until the florescent material was out of the agarose. The TEA pH8.05 buffer containing the DNA was removed from the dialysis sac and extracted once with n-butanol. The aqueous layer was dialyzed against TE pH7.2 and the sample stored at -20°C.

Rapid bacterial plasmid DNA isolation

Cells from 1.0 ml of a saturated bacterial culture were treated with lysozyme and lysed with SDS in the presence of diethyl pyrocarbonate (Davis et al., 1980), and the supernatant was ethenol precipitated. The dried ethanol precipitate was dissolved in 50 microlitres of TE pH7.2 containing 10 microgram/ml RNase A, and incubated at room temperature for 30 minutes. The reaction was stopped by the addition of 20 microlitres of dye solution (25% glycerol, 5%

SDS, 0.025% BPB) and stored at -20°C. Aliquots of 20 microlitres were used for analytical agarose gel electrophoresis.

Lambda phage DNA isolation

Phage was isolated from 1 liter of heat induced E. coli cells lysogenic for bacteriophage lambda and purified on two cesium chloride block gradients (Miller, 1972). The phage preparation was dialized against 50% formamide, 0.2M Tris, 0.02M disodium EDTA pH8.5 to disrupt the phage particles, and then against 0.1M NaCl, 0.05M Tris, 0.01M disodium EDTA (Thomas and Davis, 1974, Davis et al., 1980). The DNA was stored at -20°C.

ENZYMES

Restriction enzymes and DNA polymerase I were supplied by New England Biolabs, Beverley, Mass., or Bethesda Research Laboratories Inc., Rockville, MD. Glusulase was obtained from Endo Laboratories Inc., Garden City, N.Y. Calf intestinal alkaline phosphatase and Proteinase K were supplied by Boehringer Mannheim, and Zymolyase 5000 was obtained from Kirin Brewery Co. Ltd., Japan.

RESULTS

CONSTRUCTION OF RECOMBINANT PLASMIDS

A number of hybrid plasmids containing bacterial and yeast DNA were used in this study. They were all derivatives of pBR322 (Bolivar et al., 1977b; Sutcliff, 1978). Plasmid pBR322 has a ColE1 type origin of replication (Backman et al., 1978) and carries genes encoding resistance to the antibiotics tetracycline and ampicillin; the derivatives containing yeast DNA retain the pBR322 origin of replication and one or both of the antibiotic resistance genes.

Plasmid TLC-1 (Broach et al., 1979) was obtained from Dr. J. Hicks. It contains the yeast chromosomal regions encoding the arginine specific permease (CAN1) and beta-isopropyl malate dehydrogenase (LEU2), a fragment of the yeast plasmid Scp1 containing the origin of replication, and pBR322. The structure of TLC-1 was confirmed by restriction analysis (Appendix 1).

A number of other plasmids were constructed for use in the present study. Two derivatives of TLC-1 were made - p47-3, which contained an *in vitro* generated deletion of the CAN1 sequence and was used to transform yeast to form duplications of the chromosomal CAN1 sequences, and p36-1B,

which contained unique sequences from the LEU2 region of the yeast chromosome, and was used as a probe for these sequences. In addition, a plasmid which contained an entire copy of Scp1 was constructed. This plasmid, pTM2, was used as a source of a probe specific for sequences of Scp1 that were not part of TLC-1. The constructions of these plasmids are outlined in the following sections.

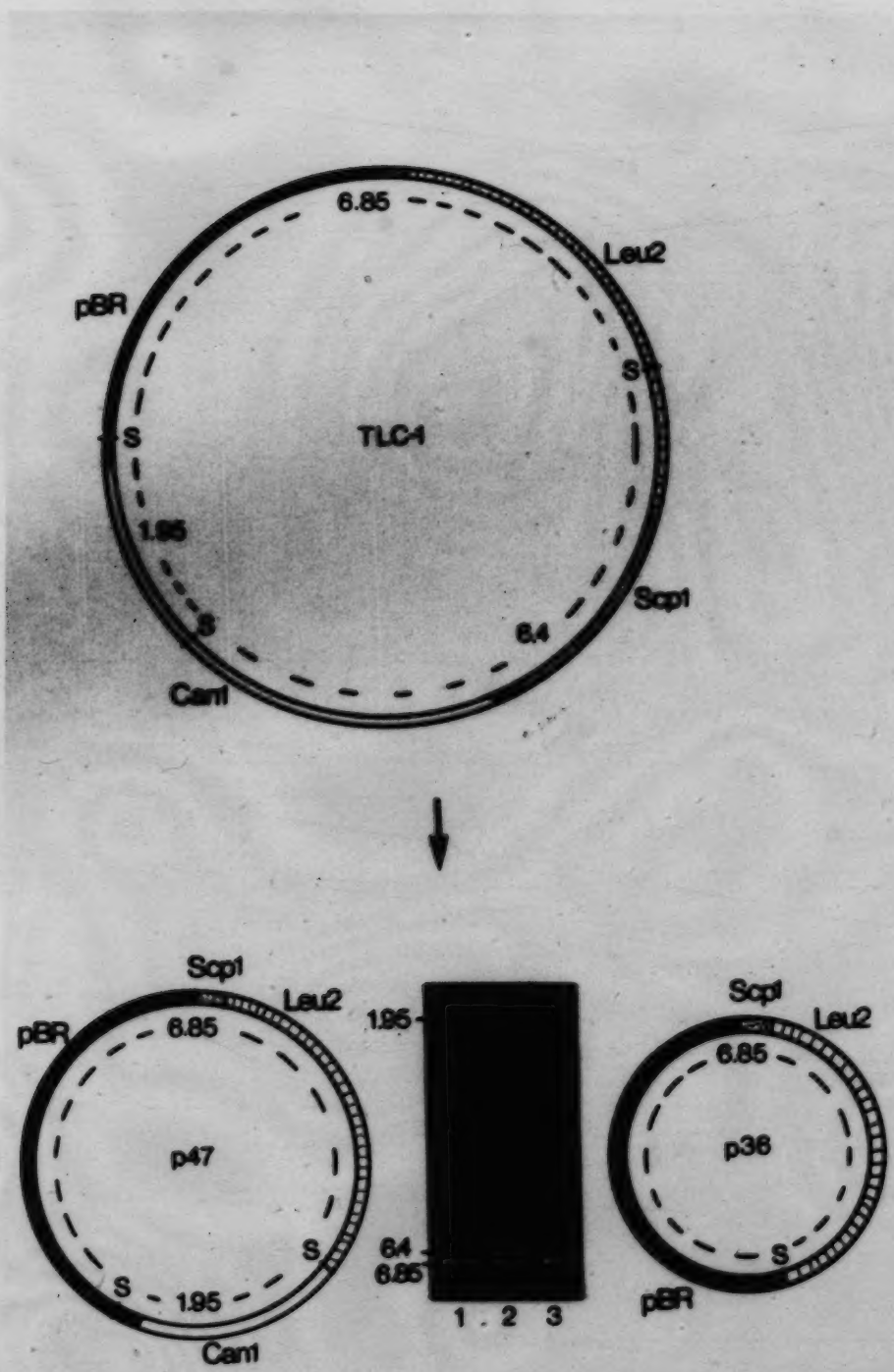
Construction of Plasmid p36-1B

Plasmid p36-1B, which contains a unique sequence from within the LEU2 fragment, but lacks the repeated elements of this region, was derived from TLC-1 in two steps. Plasmid TLC-1 was partially digested with the restriction enzyme SalI and the restriction products ligated. The ligation mix was used to transform E. coli strain JF1754 to ampicillin resistance, and the resistant transformants were tested to see if the leuB mutation of JF1754 was complemented. Rapid plasmid isolations were performed on ampicillin resistant, leucine independent colonies to identify those colonies containing plasmids smaller than TLC-1. Plasmid p36 was isolated from one of a number of independent transformants containing identically sized plasmids considerably smaller than TLC-1. It consisted of the 6.85 kb SalI fragment of TLC-1 which contained most of the pBR322 sequence, a small region of the yeast plasmid, and part of the LEU2 region (Figure 1).

Figure 1. Construction of plasmids p47 and p36

Plasmid TLC-1 was partially digested with SalI , then ligated to generate derivative plasmids p47 and p36. The line drawings show the cleavage sites for the enzyme SalI (S) and the sizes in kilobases of the SalI restriction fragments of TLC-1, p47 and p36. Sequences of the plasmids derived from pBR322 are shown in black, sequences from Scp1 are stipled, those from the LEU2 region of S. cerevisiae are hatched and those from the CAN1 region of S. cerevisiae are white (More detailed restriction maps are given in the Appendix).

The photograph shows an agarose gel on which are resolved the restriction fragments from SalI digests of (1) p47, (2) TLC-1, and (3) p36. Plasmid p47 lacks the 6.4 kb fragment found in TLC-1, plasmid p36 lacks both the 1.95 kb and 6.4 kb fragments of TLC-1.


6.85
Leu2
pBR
S
TLC1
S
1.95
S
Scp1
6.4
Can1
Scp1
Leu2
pBR
6.85
p47
S
S
1.95
Can1
1.95
6.4
6.85
1 . 2 3
Scp1
Leu2
6.85
p36
S
pBR

Further restriction analysis was used to confirm this structure (Appendix 2).

Plasmid p36 was extracted from JF1754 and purified on a CsCl/EtBr gradient. The purified plasmid was restricted to completion with EcoRI, and the restriction mix used directly to transform strain JF1754 to ampicillin resistance. A leucine requiring, ampicillin resistant colony was identified. The plasmid was isolated from this strain and purified on a CsCl/EtBr gradient. This plasmid, p36-1B, contains the pBR322 origin of replication and ampicillin resistance gene, and a unique sequence from the yeast LEU2 region (Figure 2).

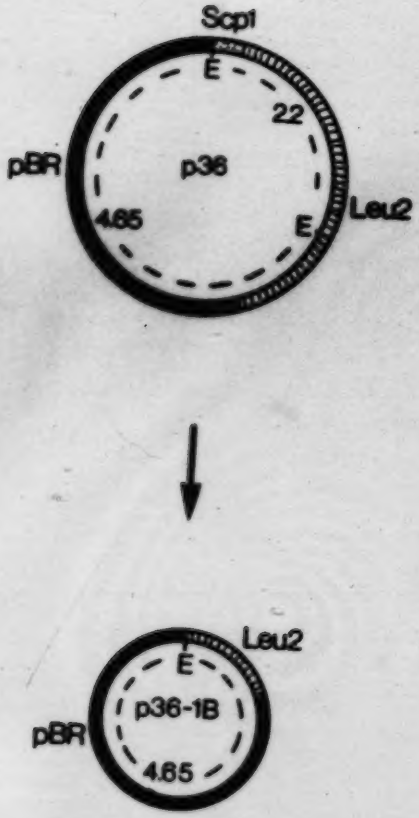
Construction of Plasmid p47-3

Plasmid p47-3, which lacks a functional CAN1 gene and yeast plasmid origin of replication, but contains a functional LEU2 gene, was also derived from TLC-1 in two steps. A colony containing a plasmid smaller than TLC-1 but larger than p36 was identified from the ampicillin resistant, leucine independent transformants generated from the partial SalI digest of TLC-1. This plasmid, p47, contains two SalI fragments; the 6.85 kb fragment also found in p36, and the 1.95 kb fragment containing part of the cloned CAN1 region (Figure 1). Further restriction analysis was used to confirm this structure (Appendix 3). Plasmid p47

Figure 2. Construction of plasmid p36-1B

Plasmid p36 was digested with EcoRI to generate derivative plasmid p36-1B. The line drawings show the cleavage sites for the enzyme EcoRI (E) and the sizes in kilobases of the EcoRI restriction fragments of p36 and p36-1B. Sequences of the plasmid derived from pBR322 are black, sequences from Scp1 are stipled, and sequences from the LEU2 region of S. cerevisiae are hatched. (See Appendix for a more detailed restriction map).

The photograph shows an agarose gel on which are resolved the restriction fragments from EcoRI digests of (1) p36 and (2) p36-1B. Plasmid p36-1B lacks the 2.2 kb EcoRI fragment of plasmid p36.


Scp1
E
22
pBR
p36
4.65
E
Leu2
Leu2
E
p36-1B
pBR
4.65

22
4.65
1
2

does not confer canavanine sensitivity to canavanine resistant cells which carry it, so p47 does not contain an intact CAN1 gene.

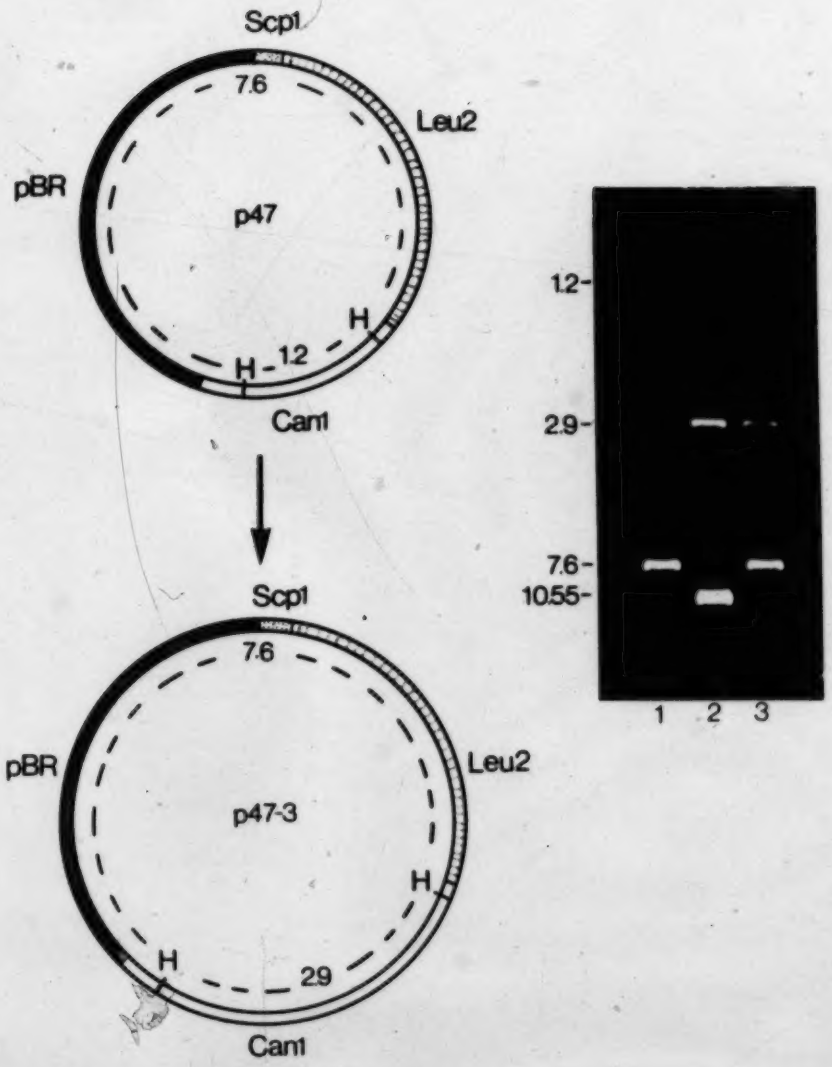
Plasmid p47 was extracted from strain JF1754 and purified on a CsCl/EtBr gradient. It was then digested to completion with HindIII and the restriction mix treated with calf intestinal alkaline phosphatase. This DNA preparation was ligated to the 2.9 kb HindIII fragment of TLC-1 that had been purified by preparative agarose gel electrophoresis. The ligation mix was used to transform JF1754 to ampicillin resistance, and rapid plasmid isolations were performed on the transformants to identify plasmids larger than p47. One such transformant, p47-3, replaced the 1.2 kb HindIII fragment of p47 with the 2.9 kb HindIII fragment from TLC-1 (Figure 3). Plasmid p47-3 was extracted from JF1754, purified on a CsCl/EtBr gradient, and subjected to restriction analysis to identify the orientation of the inserted fragment (Appendix 4).

Plasmid p47-3 does not confer canavanine sensitivity to canavanine resistant cells which carry it, so p47-3 does not contain an intact CAN1 gene.

Figure 3. Construction of plasmid p47-3

Plasmid p47-3 was constructed by ligating HindIII digested p47 to a purified 2.9 kb HindIII fragment derived from TLC-1. The line drawings show the cleavage sites for the restriction enzyme HindIII (H) and the sizes in kilobases of the HindIII fragments of p47 and p47-3. Sequences of the plasmids from pBR322 are black, sequences from Scp1 are stipled, sequences from the LEU2 region of S. cerevisiae are hatched, and sequences from the CAN1 region of S. cerevisiae are white. (Detailed restriction maps are given in the Appendix).

The photograph shows an agarose gel on which are resolved the restriction fragments from HindIII digests of (1) p47, (2) TLC-1, and (3) p47-3. Plasmid p47-3 has replaced the 1.2 kb HindIII fragment of p47 with the 2.9 kb HindIII fragment of TLC-1.


Scp1
7.6
Leu2
pBR
p47
H
H
1.2
Can1
Scp1
7.6
pBR
Leu2
p47-3
H
H
2.9
Can1
1.2-
2.9-
7.6-
10.55-
1
2
3

Construction of Plasmid pTM2

Plasmid pTM2 contains a single copy of the endogenous yeast plasmid Scp1 (Cameron et al., 1977), inserted into the unique EcoRI site of pBR322. The endogenous yeast plasmid consists of a duplex DNA circle of 6,318 nucleotide pairs (Hartley and Donelson, 1980), and has a contour length of 2 microns when measured in the electron microscope (Sinclair et al., 1967). It is often referred to as the 2 micron circle. Most strains of S. cerevisiae carry the Scp1 form of the plasmid which contains two cutting sites for the enzyme EcoRI (Cameron et al., 1977; Livingston, 1977) but some strains harbour a form of plasmid with only one cutting site for this enzyme (Livingston, 1977).

Bulk DNA was isolated from strain SS101. This strain contains the form of the plasmid with a single EcoRI cutting site (Livingston, 1977). The covalently closed circular form of the plasmid was purified from the chromosomal and mitochondrial DNA by extraction with acidified phenol (Zasloff et al., 1978). The yeast plasmid was restricted to completion with EcoRI and ligated to EcoRI cleaved pBR322. The ligation mixture was used to transform E. coli strain delta 303-2 to ampicillin resistance. Rapid plasmid isolations were used to identify transformed colonies containing plasmids larger than dimeric pBR322. Plasmid pTM2 was identified as containing an insert the same size as linear Scp1.

The yeast plasmid contains an inverted repeat of 599 nucleotide pairs (Hartley and Donelson, 1980), and exists within yeast cells in two forms resulting from intramolecular recombination between these repeats (Guerineau et al., 1976). Restriction analysis was used to identify the form and orientation of the inserted yeast plasmid. Plasmid pTM2 contains the A form of Scp1 (Hartley and Donelson, 1980), inserted into pBR322 such that the asymmetrically located unique PstI site of the yeast plasmid is closest to the PstI site of pBR322 (Appendix 6). Since only the A form of Scp1 is detected in pTM2, it is evident that recombination between the inverted repeats does not occur efficiently in E. coli.

STUDIES ON MITOTIC CHROMOSOMAL INSTABILITIES

Transformation of Yeast with Plasmid p47-3

Plasmid p47-3 lacks a yeast origin of replication and therefore cannot replicate autonomously in yeast. Transformation of a leu2 yeast strain to leucine independence using plasmid p47-3 requires that the LEU2 region of the plasmid integrate into a piece of DNA that is itself capable of replication. Such integrative plasmids (Hinnen et al., 1978) have a transformation frequency much lower than do plasmids capable of autonomous replication (Beggs, 1978; Struhl et al., 1979); p47-3 produces approximately 5 LEU2 transformants per microgram of plasmid DNA whereas TLC-1 typically produces about 5,000 LEU2 transformants per microgram of DNA (Broach et al., 1979). In addition to the true LEU2 transformants, transformation of a leu2 strain with p47-3 generates a large number of tiny leucine "independent" colonies that are leu2 when restreaked. These tiny colonies are about 100 times more common than the true LEU2 transformants.

Stability and Structure of Transformants

Strain MSW5-21A (leu2 his3 trp1), a derivative of GRF18 (leu2 his3 can1), was transformed with p47-3. One transformant from each of five independent transformations was isolated and purified. Four of the five transformants were stable for the LEU2 phenotype, and showed less than 0.5% leucine requiring cells in an overnight culture grown in non-selective liquid YEPD medium. The other transformant, MSW5-21A/p47-3 #3, had lost the LEU2 phenotype in half the cells of an overnight culture grown in liquid YEPD (Table 2).

The meiotic stability of the five transformants was tested by crossing them to Y0555-19A, a leu2 his1 trp1 tester strain. Each of the four transformants that were mitotically stable expressed the LEU2 phenotype due to a single gene - most complete tetrads segregated 2 LEU2 to 2 leu2. Transformant MSW5-21A/p47-3 #3 exhibited a cytoplasmic inheritance pattern of the LEU2 phenotype; complete tetrads segregated either 4 LEU2 to 0 leu2 or 0 LEU2 to 4 leu2 (Table 3).

The DNA from the five transformants was analyzed by transformation of E. coli. Bulk unrestricted DNA isolated from the yeast transformants was used to transform E. coli strain JF1754 to ampicillin resistance. The DNA from strain MSW5-21A/p47-3 #3 generated 100 ampicillin resistant leucine

TABLE 2

Mitotic segregation of LEU2 in transformants of MSW5-21A

Strain	Number of Colonies		%LEU2
	LEU2	leu2	
MSW5-21A/p47-3 #1	854	1	>99.9
MSW5-21A/p47-3 #2	894	0	100.0
MSW5-21A/p47-3 #3	499	470	52.0
MSW5-21A/p47-3 #4	787	0	100.0
MSW5-21A/p47-3 #5	527	0	100.0

TABLE 3

Meiotic segregation of LEU2 in transformants of MSW5-21A

Cross[1] leu2 x LEU2	Segregation of LEU2:leu2 4:0	3:1	2:2	1:3	0:4
Y0555-19A x MSW5-21A/p47-3 #1	–	–	34	–	–
Y0555-19A x MSW5-21A/p47-3 #2	–	–	9	–	–
Y0555-19A x MSW5-21A/p47-3 #3	3	–	–	–	5
Y0555-19A x MSW5-21A/p47-3 #4	1	–	8	1	–
Y0555-19A x MSW5-21A/p47-3 #5	1	–	12	1	–

[1] All 16 tetrads of the cross Y0555-19A (leu2) x MSW5-21A (leu2) segregated 0 LEU2 : 4 leu2

independent E. coli transformants per microgram of DNA. The DNA preparations from the other strains did not generate ampicillin resistant, leucine independent transformants (Table 4).

The DNA from one of the ampicillin resistant leucine independent transformants of JF1754 derived from the DNA of yeast transformant MSW5-21A/p47-3 #3 was isolated and purified on a CsCl/EtBr gradient. A plasmid band was evident in this preparation; this band was isolated and the purified plasmid DNA subjected to restriction analysis. The restriction pattern of this plasmid is consistent with it being formed by recombination between p47-3 and the endogenous yeast plasmid Scp1. This recombination event occurred within the 0.25 kb fragment, flanked by PstI and EcoRI restriction sites, that was found in both plasmids (Appendix 5).

Mapping of the Stable Transformants

Plasmid p47-3 contains two sequences derived from regions of yeast chromosomal DNA, the LEU2 region and the CAN1 region. The four stable LEU2 integrants were mapped relative to each of these regions. The LEU2 markers of the transformants were mapped relative to the normal LEU2 locus, found near the centromere of chromosome III, by crossing the transformants to X2180-1A, a LEU2 strain. All four stable transformants produced meiotic products that required

TABLE 4

Transformation of *E. coli* with DNA from *LEU2* transformants of MSW5-21A

Source of DNA	Micrograms DNA	Number of Transformants	
		Amp R[1]	*LeuB* [2]
MSW5-21A	10	0	–
MSW5-21A/p47-3 #1	10	0	–
MSW5-21A/p47-3 #2	10	0	–
MSW5-21A/p47-3 #3	10	997	50/50
MSW5-21A/p47-3 #4	10	0	–
MSW5-21A/p47-3 #5	10	0	–

[1] Ampicillin resistant

[2] Number of leucine independent colonies out of number tested

leucine; the LEU2 markers of these transformants are not linked to the LEU2 marker of strain X2180-1A (Table 5).

The LEU2 markers of the stable transformants were mapped relative to the normal CAN1 locus found on chromosome V by crossing the transformants to MSW85-15B, a leu2 can1 strain. Transformant MSWS-21A/p47-3 #1, when crossed to MSW85-15B, produced meiotic products that were either LEU2 CAN1 or leu2 can1. This cross generated parental ditypes in 28 complete tetrads that segregated 2:2 for both CAN1 and LEU2; the LEU2 marker of MSW5-21A/p47-3 #1 maps at the CAN1 locus. The other three stable transformants, when crossed to MSW85-15B, produced meiotic products that were CAN1 leu2 and can1 LEU2 as often as CAN1 LEU2 and can1 leu2; the LEU2 marker of these transformants was not linked to the CAN1 locus (Table 6).

The LEU2 markers of the four stable transformants were mapped relative to each other. Leucine independent colonies in the a mating type were selected from the meiotic products of the transformant times Y0555-19A cross, and these were crossed back to the original transformants. Each heterologous mating generated leucine requiring meiotic products; the LEU2 marker in each stable transformant maps to a location separate from the others (Table 7).

TABLE 5

Mapping integrated LEU2 of MSW5-21A transformants relative to normal chromosomal location of LEU2

Cross[1] LEU2 x LEU2	Segregation of LEU2:leu2 PD[2] 4:0	TT[3] 3:1	NPD[4] 2:2
X2180-1A x MSW5-21A/p47-3 #1	6	25	2
X2180-1A x MSW5-21A/p47-3 #2	2	5	2
X2180-1A x MSW5-21A/p47-3 #4	5	5	9
X2180-1A x MSW5-21A/p47-3 #5	3	11	5

[1] All 8 tetrads of the cross X2180-1A (LEU2) x MSW5-21A (leu2) segregated 2 LEU2 : 2 leu2

[2] Parental Ditype

[3] Tetratype

[4] Non-parental Ditype

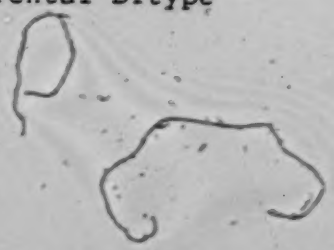


TABLE 6

Mapping integrated LEU2 of MSW5-21A transformants relative to normal chromosomal location of CAN1

Cross[1] leu2 can1 x LEU2 CAN1	Segregation of LEU2:leu2 and CAN1:can1		
	PD[2]	TT[3]	NPD[4]
MSW85-15B x MSW5-21A/p47-3 #1	28	–	–
MSW85-15B x MSW5-21A/p47-3 #2	–	10	3
MSW85-15B x MSW5-21A/p47-3 #4	2	6	–
MSW85-15B x MSW5-21A/p47-3 #5	2	4	1

[1] All 11 tetrads from the cross MSW85-15B (leu2 can1) x MSW5-21A (leu2 CAN1) segregated 2 leu2 CAN1 : 2 leu2 can1

[2] Parental Ditype (2 CAN1 LEU2 : 2 can1 leu2)

[3] Tetratype (1 CAN1 LEU2 : 1 CAN1 leu2 : 1 can1 LEU2 : 1 can1 leu2)

[4] Non-parental Ditype (2 CAN1 leu2 : 2 can1 LEU2)

TABLE 7

Mapping integrated LEU2 of MSW5-21A transformants relative to each other

Cross[1] MSW5-21A/#y LEU2 x MSW5-21A/#z LEU2	Segregation of LEU2:leu2 PD[2] 4:0	TT[3] 3:1	NPD[4] 2:2
p47-3 #1 x p47-3 #2	2	4	3
p47-3 #1 x p47-3 #4	3	5	–
p47-3 #1 x p47-3 #5	–	2	1
p47-3 #2 x p47-3 #4	1	2	2
p47-3 #2 x p47-3 #5	2	2	2
p47-3 #4 x p47-3 #5	1	9	5

[1] All homologous crosses generated only LEU2 meiotic products

[2] Parental Ditype

[3] Tetratype

[4] Non-parental Ditype

Instability of Canavanine Sensitive Phenotype of Transformants

Strain MSW5-21A/p47-3#1 has an increased frequency of production of canavanine resistant cells relative to strain MSW5-21A. Three independent cultures of MSW5-21A and MSW5-21A/p47-3 #1 were assayed for the frequency of canavanine resistant colonies per viable cell. The cultures were grown for 48 hours in liquid YEPD; suitable dilutions were plated to YEPD plates to determine viable cells, and on SC + canavanine plates to determine the number of canavanine resistant cells. There were about 50 times more canavanine resistant cells in cultures of MSW5-21A/p47-3 #1 than in cultures of MSW5-21A (Table 8).

The high frequency of production of canavanine resistant cells is linked to the LEU2 marker carried by the integrated plasmid in MSW5-21A/p47-3 #1. Strain MSW5-21A/p47-3 #1 was crossed to strain YO555-19A and the resulting diploid (D101) was sporulated. The meiotic products of 30 complete tetrads were analyzed for the production of canavanine resistant cells; 5 tetrads were assayed quantitatively, 25 were assayed qualitatively. The quantitative assay was performed by measuring the frequency of canavanine resistant cells in cultures grown for 48 hours in liquid YEPD. In each case, the frequency of canavanine resistant cells were higher in the LEU2 meiotic products (Table 9). The qualitative assay was performed by replica

TABLE 8

Appearance of *can1* colonies in cultures of MSW5-21A and in cultures of a derivative of MSW5-21A with p47-3 integrated at the *CAN1* locus

Strain	Culture	Number of Colonies			Frequency of *can1* Cells x 10^7
		YEPD[1]	SC+can[2]	SC+can[3]	
MSW5-21A	1	884	23	NT*	2.6
	2	1260	36	NT	2.9
	3	620	90	NT	14.0
MSW5-21A/ p47-3 #1	1	874	NT	247	280.0
	2	1174	NT	284	240.0
	3	563	NT	178	320.0

[1] Dilution factor 0.2 x 10^{-5} for YEPD

[2] Dilution factor 0.2 for Synthetic Complete plus canavanine

[3] Dilution factor 0.2 x 10^{-1} for Synthetic Complete plus canavanine

* Not tested

TABLE 9

Appearance of *can1* cells in cultures of *LEU2* and *leu2* meiotic products of D101

Strain	*LEU2*	YEPD[1]	SC+can[2]	SC+can[3]	Frequency of *can1* Cells x 10^7
101-1A	-	850	40	4	4.7
1B	-	585	27	2	4.6
1C	+	540	NC*	92	170.0
1D	+	797	NC	265	340.0
101-2A	-	410	30	1	7.3
2B	+	331	398	36	120.0
2C	-	506	155	15	28.0
2D	+	563	NC	180	320.0
101-3A	-	324	37	1	11.0
3B	+	396	NC	542	1400.0
3C	-	568	45	4	7.9
3D	+	585	NC	800	1400.0
101-4A	-	512	46	2	9.0
4B	+	558	NC	125	220.0
4C	+	339	642	63	190.0
4D	-	570	13	2	2.3
101-5A	-	330	17	0	5.2
5B	+	339	369	45	110.0
5C	-	455	54	3	12.0
5D	+	579	NC	107	180.0

[1] Dilution factor 0.2 x 10^{-5} for YEPD

[2] Dilution factor 0.2 for Synthetic Complete plus canavanine

[3] Dilution factor 0.2 x 10^{-1} for Synthetic Complete plus canavanine

* Not counted

plating colonies derived from the meiotic products of D101 to SC + canavanine plates. In all cases, the tetrads segregated two LEU2 colonies which generated many canavanine resistant papillae on the canavanine plates, and two leu2 spores that generated few or no canavanine resistant papillae.

The transformants that do not have p47-3 integrated at the CAN1 locus do not show any enhancement of canavanine resistant cell production associated with the LEU2 marker. Each of MSW5-21A/p47-3 #2, 4 and 5 were crossed to Y0555-19A to generate diploids D102, D104 and D105. These diploids were sporulated and a single complete tetrad was analyzed quantitatively for the frequency of canavanine resistant cells in cultures from each meiotic product. There was no significant enhancement in the frequency of canavanine resistant cells in the cultures of the LEU2 meiotic products (Table 10).

The canavanine resistant derivatives of MSW5-21A/p47-3 #1 frequently become leucine requiring. The formation of jointly leucine dependent canavanine resistant cells requires that p47-3 be integrated at the CAN1 locus, and is independent of the RAD52-1 gene. A total of 300 single colonies from each of three strains - MSW5-21A/p47-3 #1, which is RAD52 and carries p47-3 integrated at the CAN1 locus, MSW-21A/p47-3 #2, which is RAD52 and carries p47-3 integrated at a site unlinked to the CAN1 locus, and MSW75-

TABLE 10

Appearance of *can1* cells in cultures of *LEU2* and *leu2* meiotic products of D102, D104 and D105

Strain	*LEU2*	Number of Colonies YEPD[1]	SC+can[2]	SC+can[3]	Frequency of *can1* Cells x 10^7
102-1A	-	606	32	1	5.3
1B	+	545	70	8	13.0
1C	+	271	38	4	14.0
1D	-	475	26	3	5.5
104-2A	+	919	114	9	12.0
2B	-	256	25	2	9.8
2C	-	586	34	3	6.3
2D	+	10	24	2	240.0
105-1A	+	627	37	3	5.9
1B	-	696	41	4	5.9
1C	-	608	34	3	5.6
1D	+	419	43	3	10.0

[1] Dilution factor 0.2 x 10^{-8} for YEPD

[2] Dilution factor 0.2 for Synthetic Complete plus canavanine

[3] Dilution factor 0.2 x 10^{-1} for Synthetic Complete plus canavanine

2C, which is rad52-1 and carries p47-3 integrated at the CAN1 locus - were patched on SC + canavanine plates. A single canavanine resistant colony was selected from each patch which produced one or more canavanine resistant papillae. Each independent canavanine resistant derivative was tested for a leucine requirement; both MSW5-21A/p47-3#1 and MSW75-2C had about 4% of the canavanine resistant cells exhibiting a leucine requirement, while none of the canavanine resistant derivatives of MSW5-21/p47-3 #2 required leucine (Table 11).

Strain MSW5-21A/p47-3#1 generates leucine requiring segregants that are not canavanine resistant. Independent colonies of MSW5-21A/p47-3#1 were inoculated into 2.0 mls of liquid YEPD and shaken for 48 hours at 30°C. Suitable dilutions of each culture were made to produce approximately 250 colonies per YEPD plate; 5 plates were spread for each culture and the plates were incubated at 30°C for 2 days. The colonies that appeared were tested for leucine dependence by replica plating to SC-leucine plates. Fifteen independent cultures were assayed, 3 of which contained leucine requiring cells. A total of 10 leucine requiring colonies were detected in 23,000 colonies analyzed; 8 of these came from the same culture. However, these 8 leucine requiring colonies represent at least two events; 3 of the colonies were also canavanine resistant whereas the other 5 remained canavanine sensitive (Table 12).

TABLE 11

Appearance of *leu2* *can1* derivatives of strains containing an integrated p47-3

Strain	*RAD52*	p47-3 at *CAN1*	Independent Cells' *can1*	*can1* *leu2*	%*can1* *leu2*
MSW5-21A/ p47-3 #1	+	+	292	13	4.4
MSW5-21A/ p47-3 #2	+	-	137	0	-
MSW75-2C	-	+	290	11	3.8

' 300 colonies tested for each strain

TABLE 12

Isolation of *leu2* derivatives of MSW5-21A/p47-3 #1

Culture	Number of Colonies Analyzed	Number of Colonies	
		leu2 *CAN1*	*leu2* *can1*
1	1849	–	–
2	1912	1	–
3	1909	–	–
4	1850	–	–
5	1815	–	–
6	1275	–	–
7	1508	5	3
8	1452	–	–
9	1247	–	–
10	1267	–	–
11	1330	–	–
12	1340	–	–
13	1407	–	–
14	1313	1	–
15	1422	–	–
Total	22916	7	3

Further leucine requiring colonies were generated by subculturing (0.01 ml into 2.0 ml) saturated cultures of MSW5-21A/p47-3#1 twice before plating for single colonies. A total of 13 leucine requiring derivatives were detected in 22,000 colonies analyzed; 8 of these were from separate tubes and should represent independent events (Table 13).

Structure of the CAN1 locus of MSW5-21A/p47-3#1

The physical basis for the joint instability of the CAN1 and LEU2 markers of strain MSW5-21A/p47-3#1 was examined by Southern analysis(Southern, 1975). Bulk DNA was isolated from the strains of interest, restricted with EcoRI and fractionated on 1% agarose gels, and blotted to nitrocellulose. The nitrocellulose filters were hybridized with nick-translated DNA from the 2.9 kb HindIII fragment (co-ordinates 6.80 to 9.70) of TLC-1 (Appendix 1). This probe is specific for sequences of the CAN1 locus of S. cerevisiae.

Strain MSW5-21A contained a single EcoRI fragment of approximately 10 kb that had homology to the can1 probe (Figure 4A). The sizes of fragments with homology to the probe were assigned by comparing the migration distance of the band on the autoradiograph with the migration distance, on the original gel, of HindIII fragments of lambda. This method of estimation is somewhat imprecise because of the

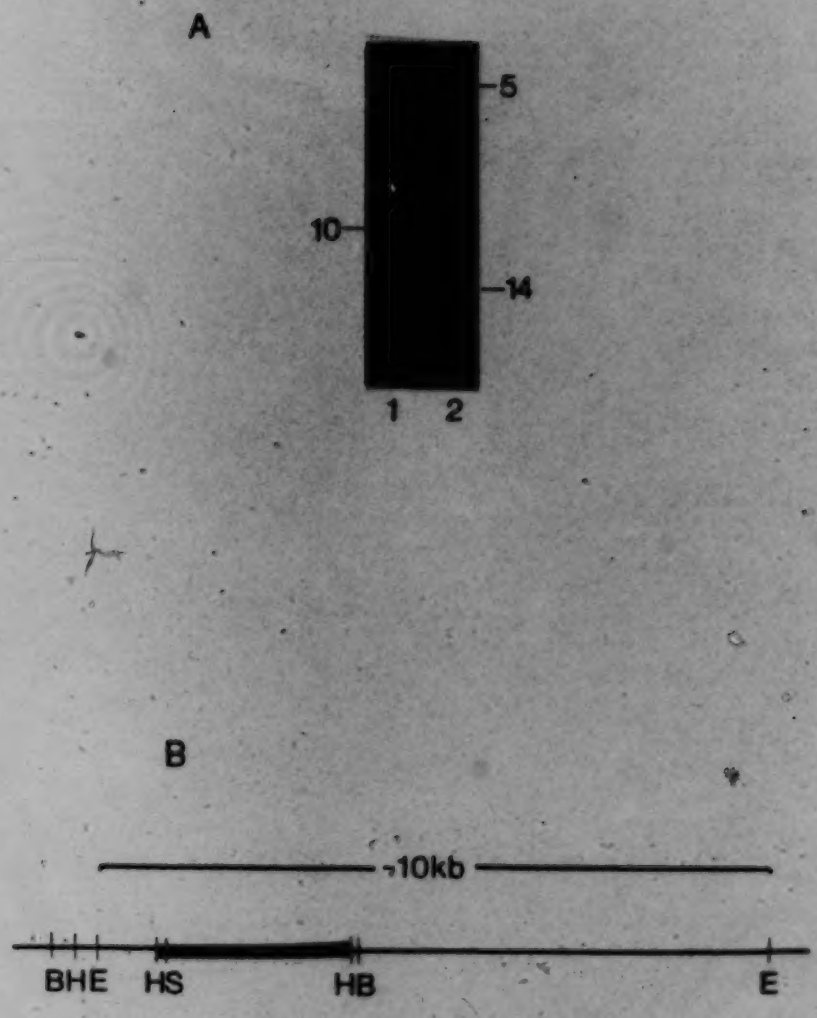
TABLE 13

Isolation of *leu2* derivatives of MSW5-21A/p47-3 #1 after subculturing

Culture	Number of Colonies Analyzed	Number of Colonies	
		leu2 CAN1	*leu2* can1
1	1469	2	–
2	1128	–	–
3	1040	1	–
4	1227	1	–
5	1362	1	–
6	995	–	–
7	668	–	–
8	552	–	–
9	489	–	–
10	601	–	–
11	528	1	–
12	2682	4	–
13	2601	2	–
14	2490	–	–
15	2314	–	–
16	2121	1	–
Total	22317	13	0

Figure 4A. Southern blot of EcoRI digested DNA from strains MSW5-21A (lane 1) and MSW5-21A/p47-3 #1 (lane 2) hybridized with a nick translated probe specific for the CAN1 region of S. cerevisiae. The approximate sizes in kilobases of the EcoRI fragments which show homology to the probe are indicated.

Figure 4B. Restriction sites in the CAN1 region of MSW5-21A. The scale is 1 cm = 1 kb. The position of the cutting sites for the restriction enzymes BamHI (B), HindIII (H), EcoRI (E), and SalI (S) are shown. The position of the right most EcoRI site is estimated from the size of the EcoRI fragment with homology to the can1 probe. The region of homology to the probe is shown by the thicker black line.


A
5
10
14
1 2
B
~10kb
BHE
HS
HB
E

possibility of the nitrocellulose shrinking during baking; sizes determined in this manner will always be designated approximate.

Only one EcoRI site is found in the 4.5 kb BamHI fragment of yeast DNA, containing the CAN1 gene, that is cloned in plasmid TLC-1. The other EcoRI site that generates the 10 kb fragment is found outside the cloned region. It is approximately 6 kb from the BamHI site, used in the cloning of the CAN1 gene, that was not reformed in TLC-1 (Figure 4B).

The genetic evidence that plasmid p47-3 had integrated at the CAN1 locus in transformant MSW5-21A/p47-3 #1 was confirmed by Southern analysis. Strain MSW5-21A/p47-3 #1 contained two EcoRI fragments showing homology to the can1 probe; one fragment was approximately 5 kb, the other approximately 14 kb (Figure 4A). Neither of the two fragments of MSW5-21A/p47-3 #1 that contain homology to the can1 probe is the same size as the fragment in MSW5-21A; duplication of the CAN1 gene in strain MSW5-21A/p47-3 #1 has disrupted the restriction pattern of the wild type CAN1 locus. Therefore, integration of p47-3 must have occurred within the approximately 10 kb fragment of yeast chromosomal DNA that contains the wild type CAN1 locus.

There are three regions of p47-3 that are also found at the CAN1 locus of S. cerevisiae. A single crossover between

p47-3 and the yeast chromosome at any of these regions of homology would integrate the plasmid at the CAN1 locus. These sequences are discussed below.

One sequence found both on the plasmid and at the chromosomal CAN1 locus occurs between coordinates 6.20 and 6.52 of p47-3. The homologous sequence on the chromosome lies outside the 10 kb EcoRI fragment which hybridizes to the can1 probe. Integration of p47-3 at this site would not disrupt the 10 kb EcoRI fragment, and would generate a second fragment, of undefined size, with homology to the probe (Figure 5A). The second region found on the plasmid and also on the chromosome lies between co-ordinates 3.30 and 6.20 of plasmid p47-3. The homologous region of the yeast chromosome is within the 10 kb EcoRI fragment that hybridizes the can1 probe. Integration of p47-3 by a single crossover within this region of homology would generate two EcoRI fragments which hybridize the can1 probe. One fragment would be 4.9 kb, the other approximately 13.5 kb (Figure 5B).

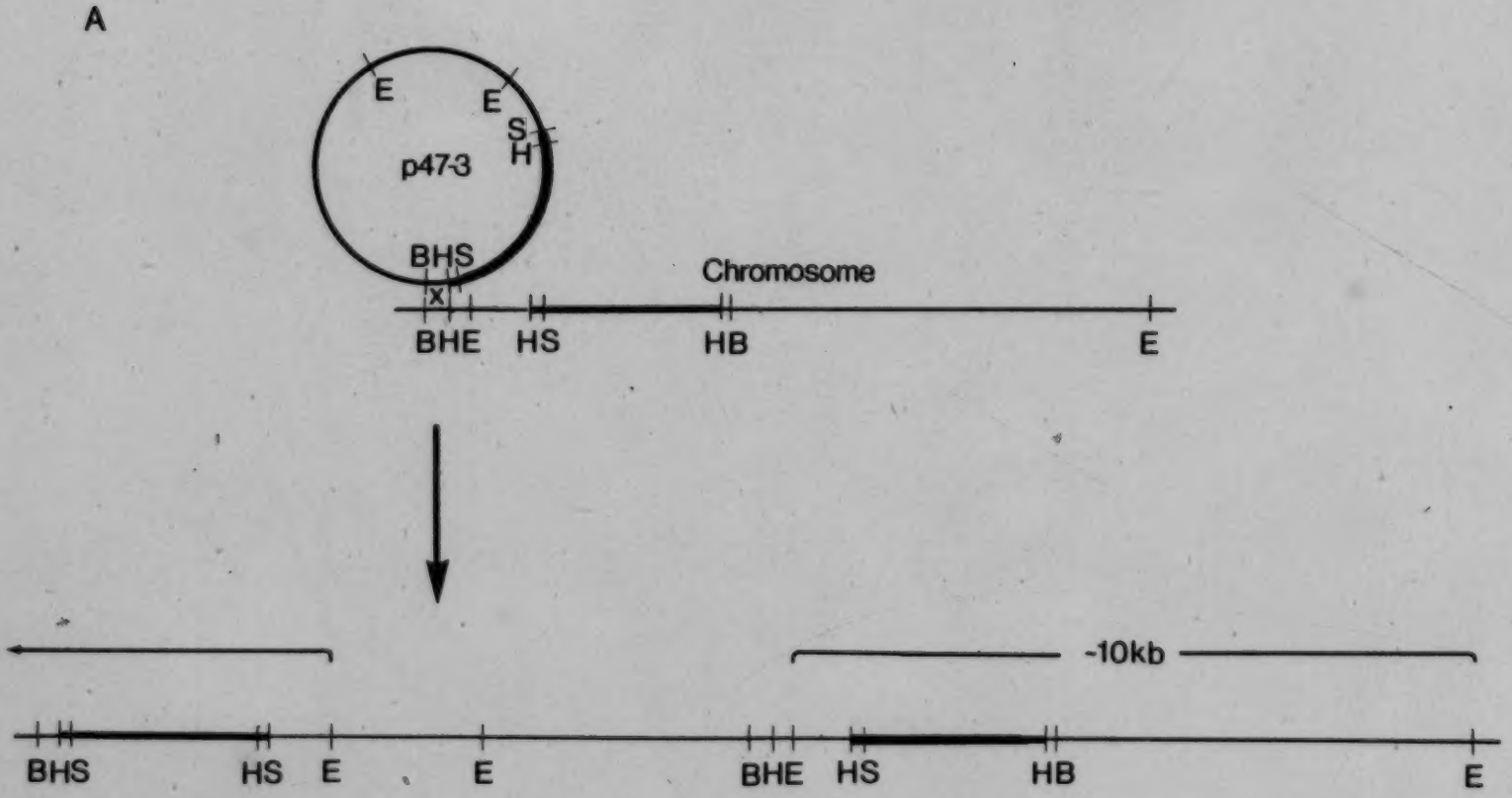
The final region found both on p47-3 and at the can1 locus occurs between co-ordinates 3.15 and 3.30 of p47-3. The homologous sequence on the chromosome occurs within the 10 kb EcoRI fragment which hybridizes to the can1 probe. Integration of p47-3 at this site would generate two EcoRI fragments with homology to the can1 probe; one of 2.0 kb, and one of approximately 16.5 kb (Figure 5C). The sizes of

Figure 5. The integration of plasmid p47-3 into the chromosomal *CAN1* region of MSW5-21A. The cutting sites for the enzymes *BamHI* (B), *HindIII* (H), *EcoRI* (E), and *SalI* (S), regions of homology to the *can1* probe (thick black line) and the sizes of the *EcoRI* fragments with homology to this probe are shown. The scale is 1 cm = 1 kb.

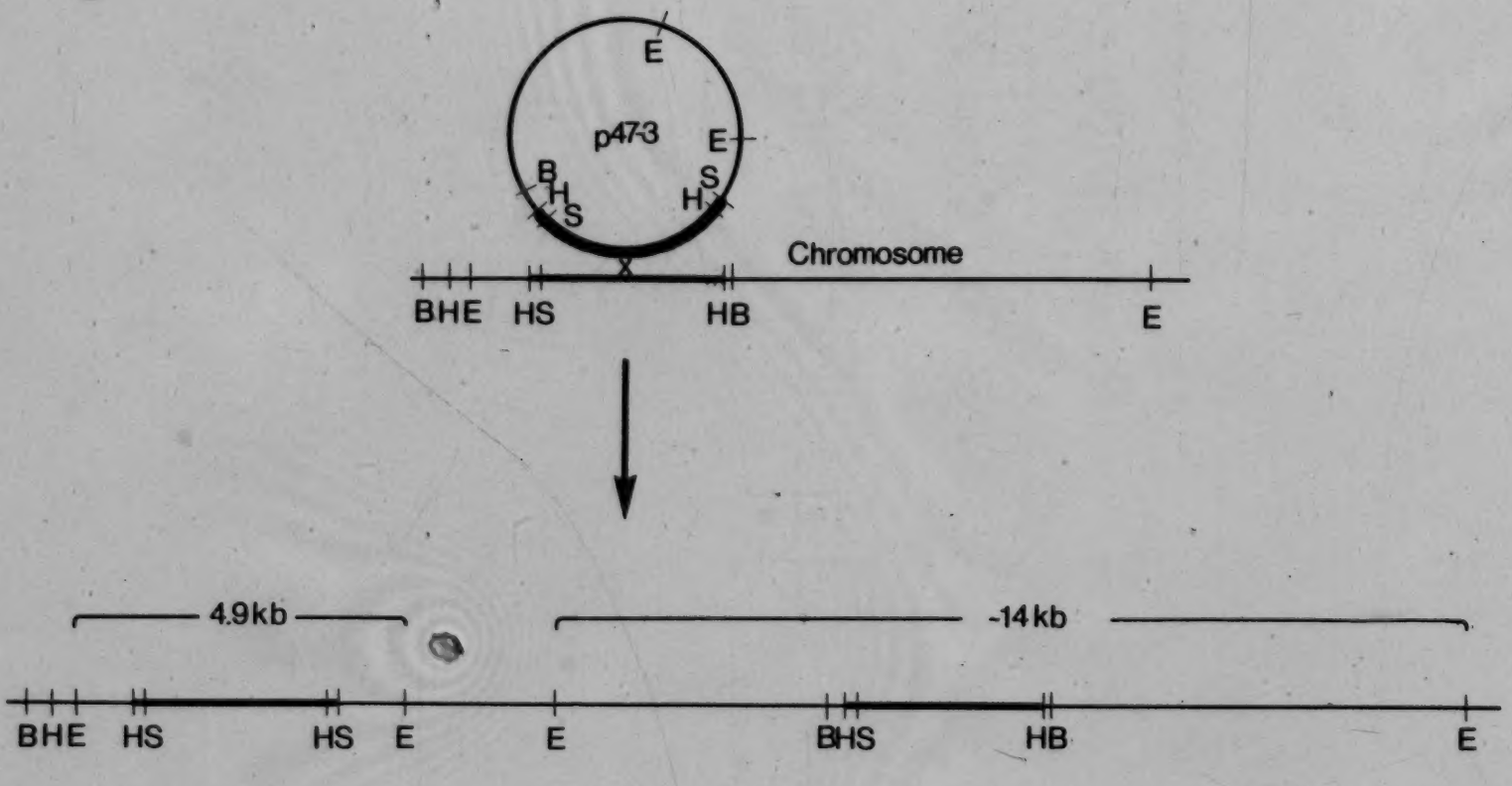
A - Integration of p47-3 by a crossover within the *BamHI* - *HindIII* fragment of the plasmid and the chromosome.

B - Integration of p47-3 by a crossover within the *HindIII* fragment of the plasmid and the chromosome.

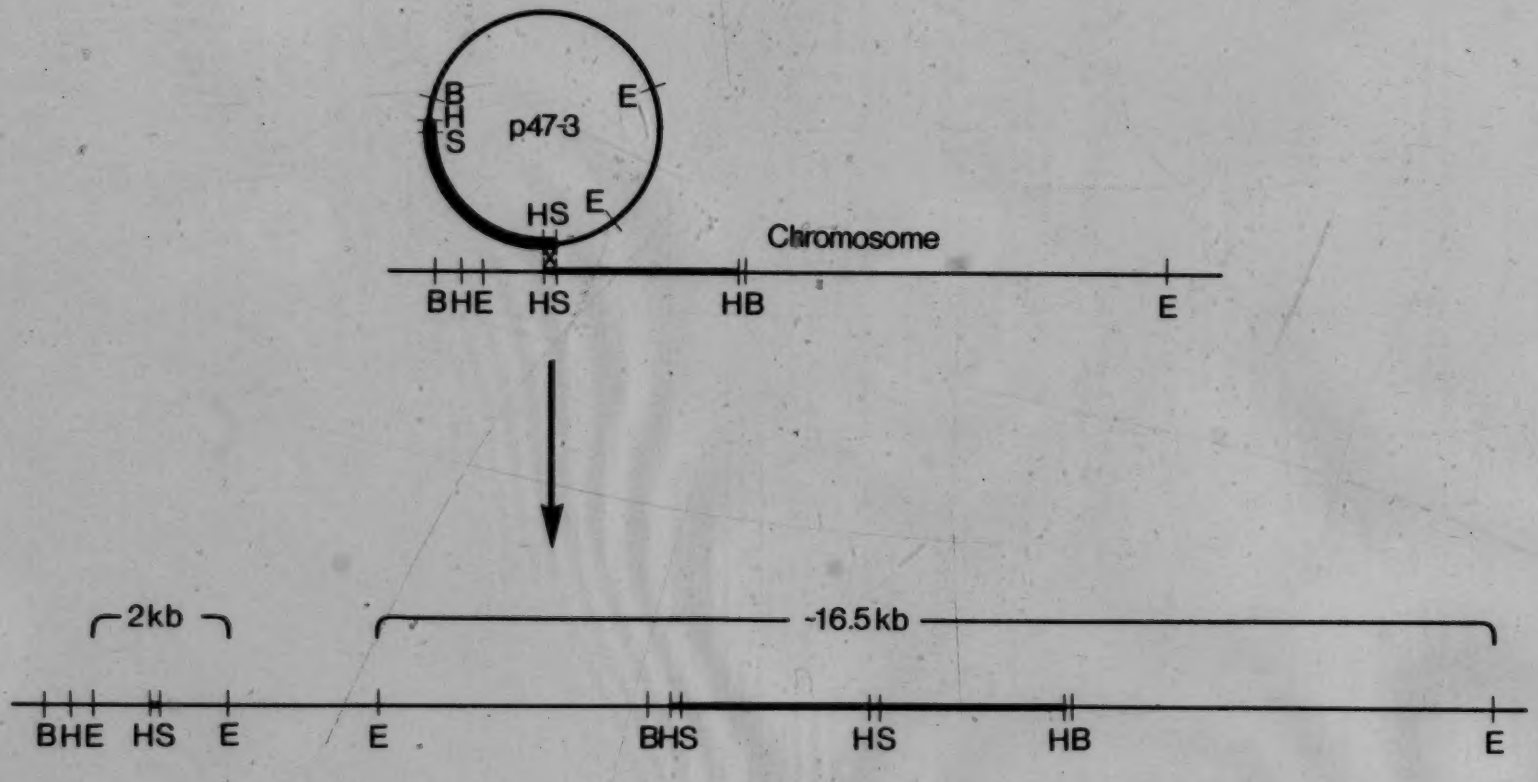
C - Integration of p47-3 by a crossover within the *HindIII* - SalI fragment of the plasmid and the chromosome.


A
E
E
S
H
p47-3
BHS
X
Chromosome
BHE
HS
HB
E
~10kb
BHS
HS
E
E
BHE
HS
HB
E

B


E
p47-3
E
B
H
S
S
H
Chromosome
BHE
HS
HB
E
4.9kb
-14kb
BHE
HS
HS
E
E
BHS
HB
E

C

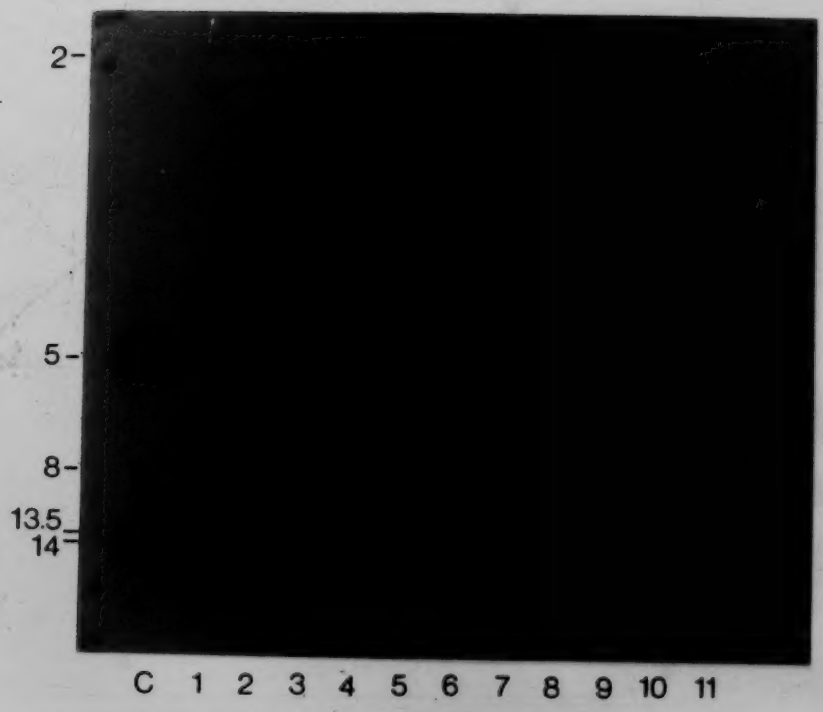

B
H
S
p47-3
E
HS
E
Chromosome
BHE
HS
HB
E
2kb
~16.5kb
BHE
HS
E
E
BHS
HS
HB
E

the EcoRI fragments in MSW5-21A/p47-3#1 that hybridize the can1 probe are consistent with p47-3 integrating by a single crossover within the 2.9 kb sequence of homology between the plasmid and the chromosome as shown in Figure 5B.

Physical Analysis of Instability of MSW5-21A/p47-3#1

The physical structure of the CAN1 locus in derivatives of MSW5-21A/p47-3 #1 that had become either canavanine resistant, or leucine requiring, or both canavanine resistant and leucine requiring was analyzed by Southern blotting. Twenty two independent canavanine resistant derivatives were analyzed; one of these had also become leucine requiring. These derivatives fall into four classes. In the majority of the canavanine resistant derivatives (16/22), the 5 kb EcoRI fragment with homology to the can1 probe had been replaced by a smaller EcoRI fragment of approximately 2 kb. The next most frequent class (4/22) had also replaced the 5 kb fragment, this time with a new EcoRI fragment of approximately 8 kb that contained homology to the can1 probe. In one canavanine resistant strain the pattern was unchanged from that found in MSW5-21A/p47-3 #1. The strain that had become jointly canavanine resistant and leucine requiring contained a single EcoRI fragment of approximately 14 kb that contained homology to the can1 probe (Figure 6). Eleven independent leucine requiring strains were analyzed; one of these had also become

Figure 6A. Southern blots of EcoRI digested DNA from strain MSW5-21A/p47-3 #1 (lanes C) and can1 derivatives of MSW5-21A/p47-3 #1 (lanes 1-22), hybridized with nick-translated can1 probe. Derivative 6 has become jointly can1 and leu2. The ordinate values are the approximate size in kilobases of the EcoRI fragments with homology to the can1 probe. In this autoradiograph the exposure was not sufficient to show clearly the 2.0 kb fragment with homology to the can1 probe (See Figure 6B).


2
5
8
13.5
14
C 1 2 3 4 5 6 7 8 9 10 11

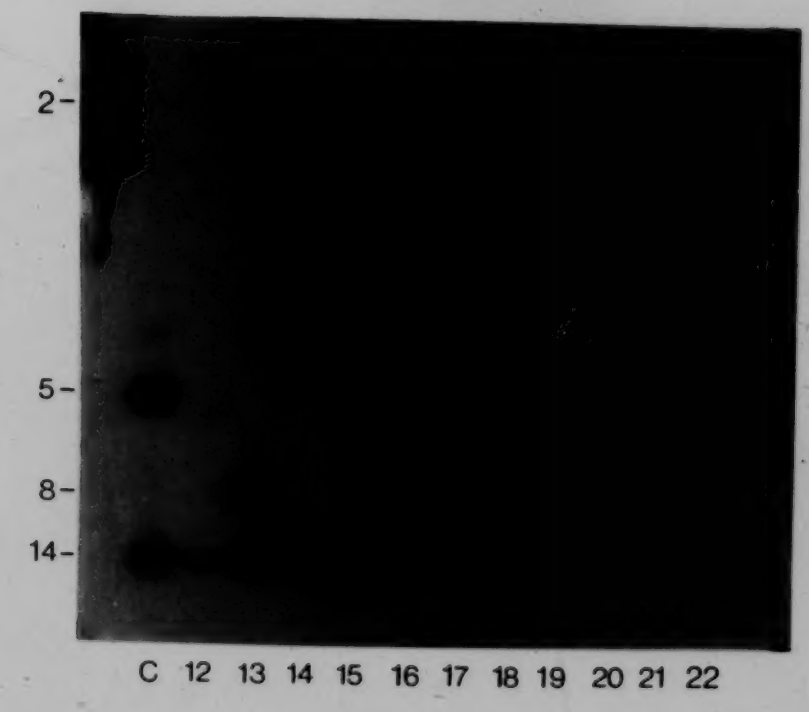
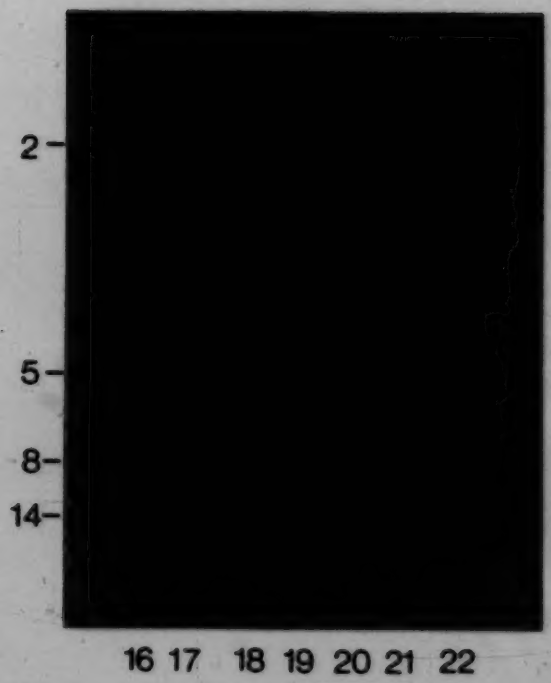

2
5
8
14
C 12 13 14 15 16 17 18 19 20 21 22

Figure 6B. Southern blot of EcoRI digested DNA from can1 derivatives 16 to 22 of strain MSW5-21A/p47-3 #1, hybridized with nick-translated can1 probe. The ordinate values are the approximate size in kilobases of the EcoRI fragments with homology to this probe. This autoradiograph was overexposed to visualize the 2.0kb EcoRI fragment with homology to the can1 probe that can be seen in derivatives 16, 18, 20, 21 and 22.


2
5
8
14
16 17 18 19 20 21 22

canavanine resistant. All these strains contained a single EcoRI fragment with homology to the can1 probe. The majority of the leucine requiring derivatives (6/11) contained a fragment with homology to the can1 probe of approximately 10 kb, identical in size to the EcoRI fragment in untransformed MSW5-21A. The remaining leucine requiring, canavanine sensitive strains contained an approximately 14 kb EcoRI fragment that hybridized the can1 probe, as did the leucine requiring, canavanine resistant strain (Figure 7).

Resolution of Non-contiguous Direct Repeats

The majority of the structural rearrangements found in derivatives of MSW5-21A/p47-3#1 can be explained by events that resolve a non-contiguous direct repeat into a single copy of the duplicated sequence. These events result in the loss of one copy of the duplication together with the DNA between the duplicated elements. There are several non-contiguous directly duplicated sequences at the CAN1 locus of MSW5-21A/p47-3 #1 (Figure 8A). The 0.15 kb sequence between the HindIII and SalI restriction enzyme cutting sites (a) is repeated three times. The 2.9 kb sequence between the HindIII cutting sites (b) is repeated twice, as is the 0.325 kb sequence between the BamHI and HindIII cutting sites (c). Events which resolve each non-contiguous direct repeat have been detected.

Resolution of the a1/a2 duplication will delete a large

Figure 7. Southern blot of EcoRI digested DNA from strains MSW5-21A and MSW5-21A/p47-3 #1 (mixed in lane C), and leu2 derivatives of MSW5-21A/p47-3 #1 (lanes 1-11) hybridized with nick translated can1 probe. Derivative 11 had become jointly can1 and leu2. The ordinate values are the approximate size in kilobases of the EcoRI fragments with homology to the can1 probe.

5
10
13
14
C 1 2 3 4 5 6 7 8 9 10 11

Figure 8. Structure of the CAN1 locus in strain MSW5-21A/p47-3 #1 and in various physically rearranged derivatives. The restriction sites for the enzymes BamHI (B), HindIII (H), EcoRI (E), and SalI (S) are shown. The regions a1, a2 and a3 represent direct repeats of an approximately 150 bp sequence, b1 and b2 represent direct repeats of a 2.9 kb sequence corresponding to the can1 probe, and c1 and c2 represent direct repeats of an approximately 325 bp sequence. The thick lines in the drawings of the rearranged derivatives represent the regions showing homology to the can1 probe. The values in kilobases represent the approximate size of the EcoRI restriction fragments that contain homology to the can1 probe. The scale is 1 cm=0.5 kb.

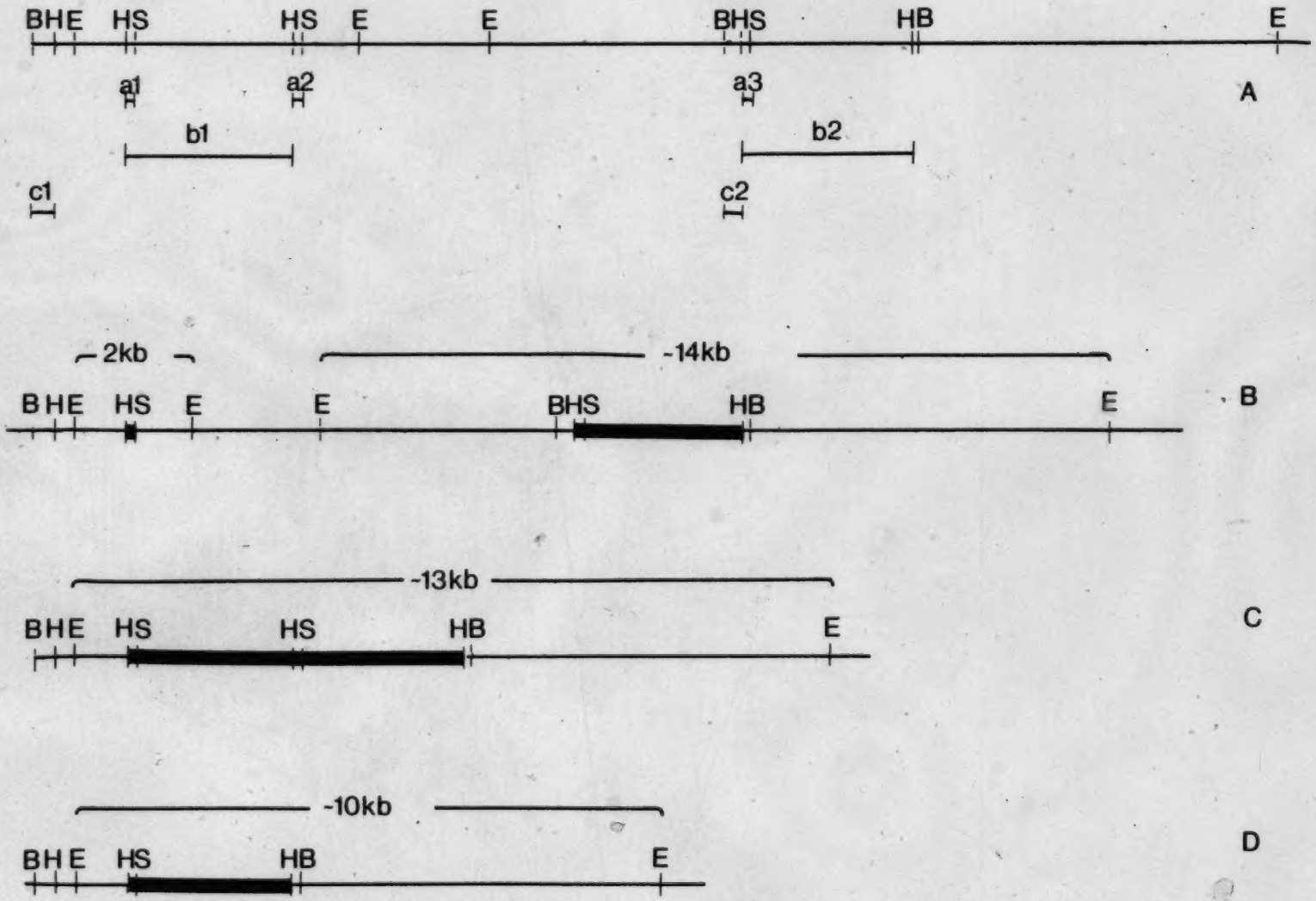
A - Original structure of the CAN1 locus in strain MSW5-21A/p47-3 #1.

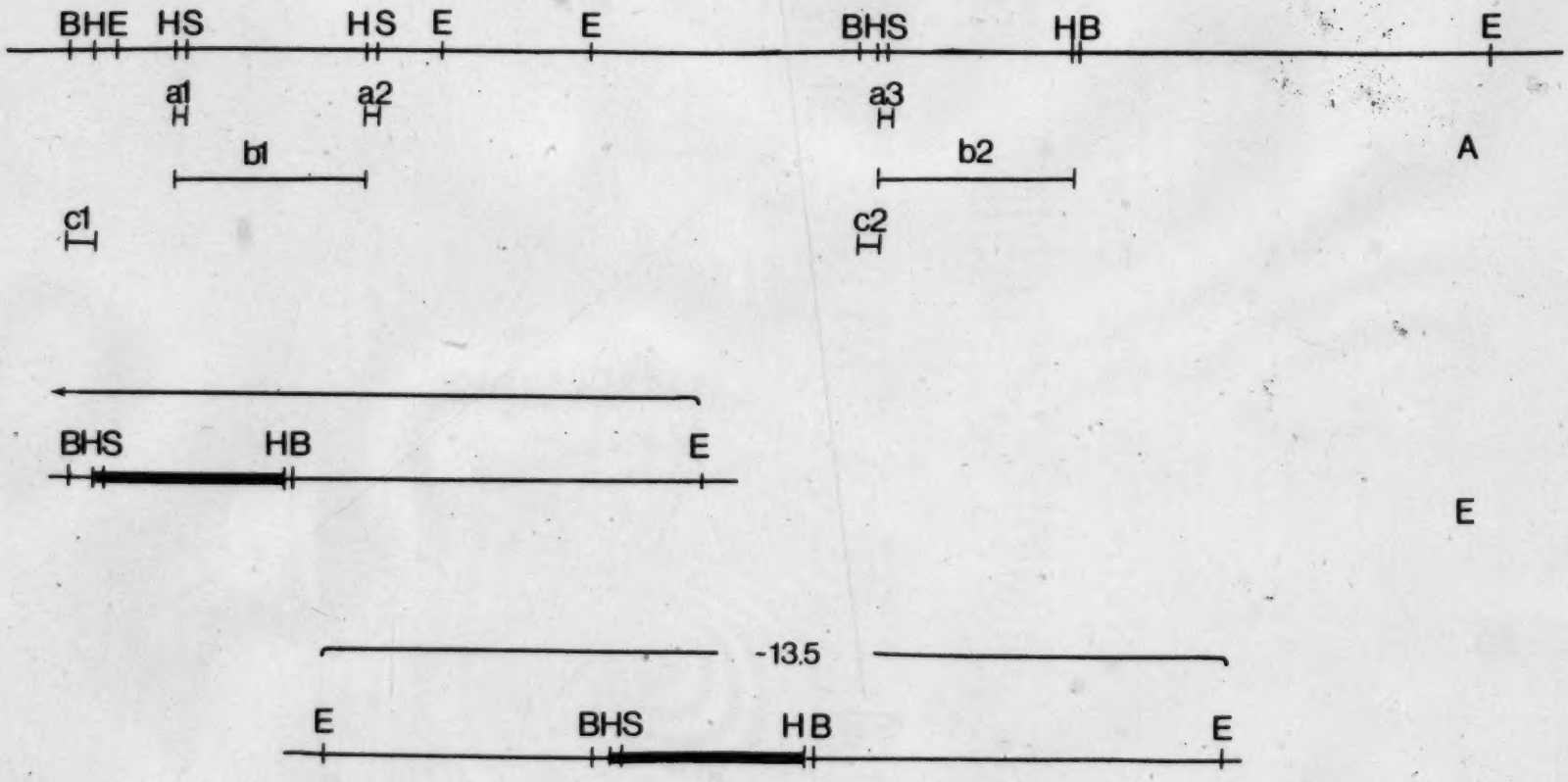
B - Structure resulting from reciprocal recombination between a1 and a2. The strains containing this structure are can1 LEU2.

C - Structure resulting from reciprocal recombination between a2 and a3. The strains containing this structure are CAN1 leu2.

D - Structure resulting from reciprocal recombination between b1 and b2. The strains containing this structure are CAN1 leu2.

E - Structure resulting from reciprocal recombination between c1 and c2. The strains containing this structure are can1 leu2.


B H E HS HS E E B HS HB E
a1 a2 a3
A
b1 b2
c1 c2
2kb
~14kb
B H E HS E E B HS HB E
B
~13kb
B H E HS HS HB E
C
~10kb
B H E HS HB E
D


BHE
HS
HS
E
E
BHS
HB
E
a1
a2
a3
b1
b2
A
c1
c2
BHS
HB
E
E
-13.5
E
BHS
HB
E

portion of the structually intact CAN1 gene of MSW5-21A/p47-3 #1, and will produce a can1 LEU2 strain. This event will reduce the 4.9 kb EcoRI which hybridizes to the can1 probe to 2.0 kb (Figure 8B). This structure was observed in 16 of 21 independent can1 LEU2 derivatives.

Resolution of the a2/a3 duplication will delete the majority of the DNA of the integrated p47-3, including the LEU2 region, but will leave the CAN1 gene intact, generating a CAN1 leu2 strain. This event will produce a 13 kb EcoRI fragment that hybridizes to the can1 probe (Figure 8C). This was found in 4 of 10 independent leu2 CAN1 derivatives.

Resolution of the a1/a3 duplication and the b1/b2 duplication are indistinguishable events. They result in a direct reversal of the integration of p47-3, and generate a CAN1 leu2 strain containing a 10 kb EcoRI fragment with homology to the can1 probe (Figure 8D). This event was detected in 6 of 10 independent leu2 CAN1 derivatives.

Resolution of the c1/c2 duplication will result in a derivative of MSW5-21A that has replaced the wild type CAN1 sequence with the sequence from p47-3. The CAN1 sequence on the plasmid is missing the 1.2 kb HindIII fragment that includes the EcoRI site of the cloned 4.5 kb BamHI fragment containing the CAN1 gene. None of the other sequences of p47-3 are left at the CAN1 locus; the overall result is the replacement of the wild type allele with an *in vitro*

generated mutant allele, a process termed transplacement (Scherer and Davis, 1979). Because the deletion of the 1.2 kb HindIII fragment removes the EcoRI site in the cloned DNA, the size of the EcoRI fragment with homology to the can1 probe in can1 leu2 derivatives of MSW5-21A/p47-3 #1 will be defined by the next EcoRI site on the chromosome (Figure 8E). Both leu2 can1 derivatives contain a single EcoRI fragment with homology to the can1 probe; this fragment is slightly smaller than the approximately 14 kb fragment found in MSW5-21A/p47-3 #1. The EcoRI site outside the 4.5 kb BamHI fragment containing the CAN1 gene is just less than 4 kb from the BamHI site, flanking the CAN1 gene, that was reformed in TLC-1 (Figure 8E).

Mutation and Gene Conversion

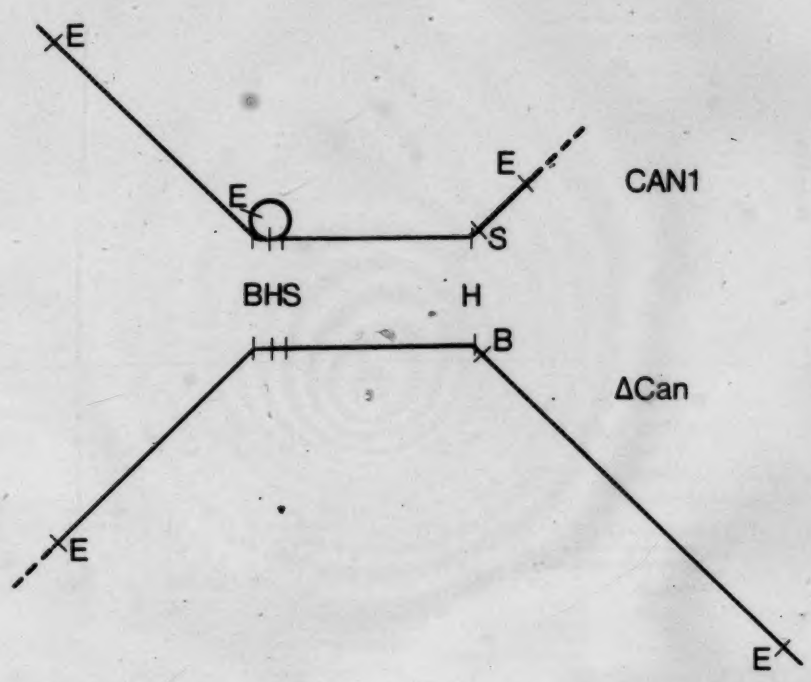
Two patterns of the Southern blots of the LEU2 can1 derivatives are not explained by events that resolve a non-contiguous direct repeat. One LEU2 can1 strain had the same structure at the CAN1 locus as MSW5-21A/p47-3 #1. This strain presumably arose by mutational inactivation of the wild type CAN1 locus. In four other strains, the 5 kb fragment with homology to the can1 probe was replaced by an approximately 8 kb fragment. This pattern can be explained by intrachromosomal gene conversion (Jackson and Fink 1981; Klein and Petes, 1981) that replaces the wild type allele of the CAN1 gene with the mutant allele containing the 1.2 kb

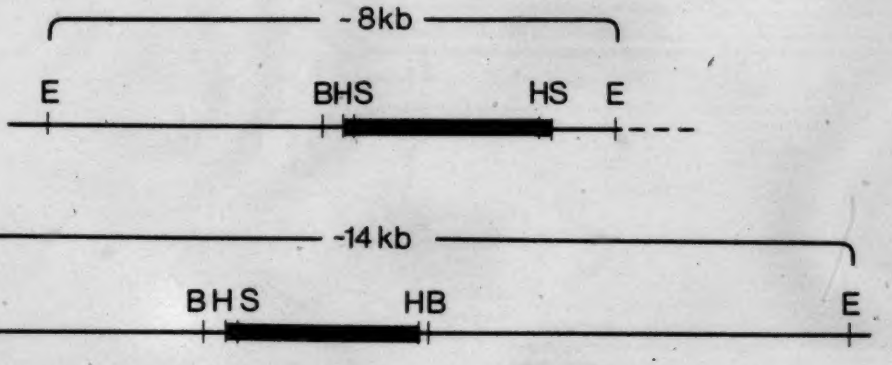
deletion. This conversion does not remove any of the duplicated sequences produced by the integration of p47-3. The 1.2 kb deletion removes an EcoRI site, so the replacement of the wild type allele of the CAN1 gene with the allele with the deletion results in a larger EcoRI fragment that contains homology to the can1 probe (Figure 9).

Effect of rad52-1 on Instability at the CAN1 Locus

The introduction of the recombination mutant rad52-1 increases the frequency of canavanine resistant cells of strains that contain a wild type CAN1 allele, but not in strains that contain p47-3 integrated at the CAN1 locus. Strain MSW72-10C, which was CAN1 rad52-1 leu2, was crossed to MSW5-21A/p47-3 #1 to generate the diploid D75. This diploid was sporulated, and the asci dissected and scored for the phenotype of the meiotic products. One complete tetrad that segregated rad52-1 and leu2 in the parental configuration, one tetrad that segregated rad52-1 and leu2 in the non-parental configuration, and 3 tetrads that segregated rad52-1 and leu2 in the tetratype configuration were selected for further analysis. Each meiotic product was inoculated into 2.0 ml liquid YEPD and shaken for 48 hours at 30°C. The frequency of canavanine resistant cells in each culture was determined by plating suitable dilutions of the cultures to YEPD and SC + canavanine plates. The leu2

Figure 9. Gene conversion between the wild-type CAN1 sequence and the deletion sequence. The top drawing shows the regions of the chromosomes aligned at their sites of homology. The bottom drawing shows the products of the conversion event that replaces the wild-type sequence with the deleted sequence. The regions of homology to the can1 probe are shown as thick lines, and the sizes in kilobases of the EcoRI fragments with homology to this probe are indicated. The dotted lines represent sequences that are contiguous on the chromosome but separate on the drawings. The restriction sites for EcoRI (E), BamHI (B), HindIII (H), and SalI (S) are also shown.


E
E
E
S
CAN1
BHS
H
B
ΔCan
E
E


~8kb
E
BHS
HS
E
~14 kb
E
BHS
HB
E

strains have a wild type CAN1 locus; the rad52-1 leu2 cultures contain about 20 times more canavanine resistant cells than do cultures of the RAD52 leu2 strains. The LEU2 strains contain p47-3 integrated at the CAN1 locus; the rad52-1 LEU2 and RAD52 LEU2 cultures contain about the same number of canavanine resistant cells (Table 14).

A single rad52-1 LEU2 strain was selected for more extensive measurements on the frequency of canavanine resistant cells. Five independent cultures of strain MSW75-2C were assayed for the frequency of canavanine resistant cells; the average frequency of can1 cells was about 3.4×10^{-5} (Table 15). As previously noted the proportion of derivatives of strains containing p47-3 integrated at the CAN1 locus with the joint phenotype leu2 can1 is not affected by the introduction of rad52-1. Both RAD52 and rad52-1 strains have about 4% of the can1 derivatives also leu2 (Table 11).

Although the overall frequency of can1 LEU2 and can1 leu2 derivatives of strains carrying p47-3 integrated at the CAN1 locus is not affected by the introduction of rad52-1, the distribution of events that generate can1 LEU2 cells is changed. Nine independent canavanine resistant derivatives of strain MSW75-2C were selected, one of these had also become leucine requiring. The structure of the CAN1 locus in these derivatives was analyzed by Southern blotting. All eight can1 LEU2 strains had two EcoRI fragments, of

TABLE 14

Appearance of canavanine resistant cells in RAD+ and rad52-1 strains with either a wild type or duplicated CAN1 locus

Strain	LEU2	RAD52	Number of Colonies YEPD[1]	SC+can[2]	SC+can[3]	Frequency of can1 Colonies x 10^7
1A	-	+	489	11	4	2.2
1B	+	-	363	616	98	270.0
1C	-	-	454	314	21	69.0
1D	+	+	629	840	99	160.0
2A	+	-	648	NC*	257	400.0
2B	-	-	212	271	33	130.0
2C	+	+	253	406	50	160.0
2D	-	+	447	25	5	5.2
3A	-	+	609	18	2	3.5
3B	-	-	308	526	46	150.0
3C	+	-	206	157	42	200.0
3D	+	+	439	861	97	220.0
10A	+	+	108	130	12	120.0
10B	+	+	184	513	55	280.0
10C	-	-	272	438	47	160.0
10D	-	-	266	367	42	140.0
15A	+	-	351	NC	87	250.0
15B	+	-	195	87	26	45.0
15C	-	+	616	79	7	13.0
15D	-	+	359	16	3	4.4

Average frequencies x 10^7:

RAD52 leu2 5.7 — rad52-1 leu2 130.0

RAD52 LEU2 240.0 — rad52-1 LEU2 185.0

[1] Dilution factor 0.2 x 10^{-5} for YEPD

[2] Dilution factor 0.2 for Synthetic Complete plus canavanine

[3] Dilution factor 0.2 x 10^{-1} for Synthetic Complete plus canavanine

* Not counted

TABLE 15

Appearance of *can1* cells in cultures of a *rad52-1* strain containing p47-3 integrated of the *CAN1* locus

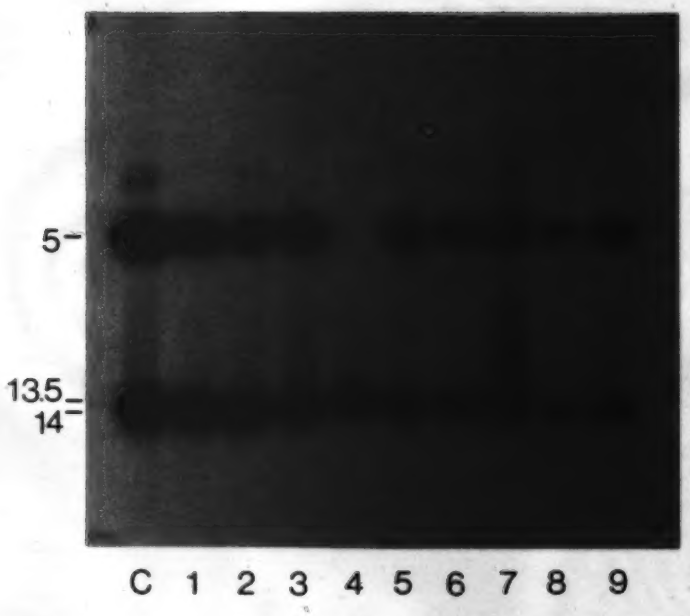
Culture	Number of Colonies YEPD[1]	Number of Colonies SC+can[2]	Frequency of *can1* Cells x 10^5
1	620	234	3.8
2	546	398	7.3
3	619	103	1.7
4	630	141	2.2
5	685	88	1.3

[1] Dilution factor 0.2×10^{-5} for YEPD

[2] Dilution factor 0.2×10^{-1} for Synthetic Complete plus canavanine

approximately 5 kb and 14 kb, that hybridized to the can1 probe. These are identical in size to the fragments observed in the original transformant. Therefore these can1 LEU2 derivatives presumably arose by mutational inactivation of the wild type CAN1 locus. The can1 leu2 strain had a single EcoRI fragment of just less than 14 kb that contained homology to the can1 probe (Figure 10).

Figure 10. Southern blot of EcoRI digested DNA from strain MSW75-2C (lane C) and can1 derivatives of MSW75-2C (lanes 1-9), hybridized with nick-translated can1 probe. Derivative 4 has become jointly can1 and leu2. The ordinate values are the approximate size in kilobases of the EcoRI fragments with homology to the can1 probe.


5
13.5
14
C 1 2 3 4 5 6 7 8 9

INSTABILITY OF PLASMID TLC-1

The specific permease for arginine uptake in S. cerevisiae is encoded by the CAN1 gene (Grenson et al., 1966). This gene determines sensitivity to high levels of the arginine analogue canavanine; strains with a functional permease are sensitive to an external concentration of 60 mg/ml canavanine sulphate, whereas strains carrying a non-functional permease are resistant (Fink, 1970). Mutant alleles are recessive to the wild type allele at the CAN1 locus, as would be expected for lesions within the structural gene for the permease.

The wild type allele of CAN1 has been cloned using the yeast and E. coli hybrid vector YEp13 (Broach et al., 1979). The plasmid containing the CAN1 gene has this gene inserted into the unique BamHI restriction site of YEp13. This plasmid, TLC-1, will efficiently transform leu2 can1 yeast strains to LEU2 and CAN1, and E. coli leuB6 strains to leucine independence and ampicillin resistance (Broach et al., 1979).

Replicational Instability of TLC-1

Yeast cells do not stably maintain plasmid TLC-1. Single colonies of strains MSW28-10C and GRF18 transformed with TLC-1 were isolated on plates lacking leucine. These colonies were inoculated into 2.0 ml aliquots of liquid YEPD medium and shaken at 30°C for 24 hours. The proportion of cells within the population that had lost TLC-1 was determined by plating a suitable dilution of each culture on YEPD plates and replicating the colonies obtained after 2 days growth at 30°C to plates lacking leucine. Approximately half the cells in an overnight culture had lost the plasmid and had become leucine requiring (Table 16).

The mitotic stability of TLC-1 is reduced in strains that carry mutations at the RAD52 locus. Plasmid TLC-1 was introduced into a rad52-1 leu2 can1 strain by crossing a leu2 can1 strain transformed with TLC-1, to a LEU2 CAN1 rad52-1 strain, and isolating a rad52-1 meiotic product that was unstable for LEU2 and CAN1 , designated MSW152-1A [TLC-1]. Colonies of this strain, inoculated from plates lacking leucine into liquid YEPD, lose TLC-1 from about 70% of the cells in the cultures (Table 17).

The endogenous yeast plasmid Scp1 greatly affects the mitotic stability of plasmid TLC-1. Strains which contain Scp1 maintain TLC-1 in a much higher proportion of cells than do strains which lack Scp1. Strain GRF18 will maintain

TABLE 16

Mitotic stability of plasmid TLC-1 in RAD$^+$ cir$^+$ strains grown in liquid YEPD

Strain	Culture	Number of Colonies		%LEU2
		LEU2	leu2	
MSW28-10C[TLC-1]	1	302	222	58
	2	145	141	51
	3	165	161	51
	4	196	135	59
	5	175	215	45
GRF18[TLC-1]	1	60	76	44
	2	80	70	53
	3	47	77	38
	4	38	56	40
	5	87	71	55

TABLE 17

Mitotic stability of plasmid TLC-1 in rad52-1 cir° strains grown in liquid YEPD

Strain	Culture	Number of Colonies		%LEU2
		LEU2	leu2	
MSW152-1A[TLC-1]	1	32	85	27
	2	54	104	34
	3	51	106	32
	4	34	92	27
	5	13	100	12

TLC-1 in about 50% of the cells of an overnight culture grown under non-selective conditions. Under identical conditions, strain YT6-2-1L, a derivative of AH22 lacking Scp1 (Erhart and Hollenberg, 1981), maintains TLC-1 in fewer than 1% of the cells (Table 18).

Even growth under selective conditions does not result in an efficient maintenance of TLC-1 in strains that lack Scp1. Single colonies of YT6-2-1L [TLC-1] were selected on synthetic complete (SC) plates lacking leucine and inoculated into 2.0 ml aliquots of liquid SC-leucine. These cultures were grown at 30°C for 48 hours and then suitable dilutions were spread on SC plates to determine the number of viable cells, and SC-leucine plates to determine the number of cells that maintained the LEU2 marker. About 5% of the viable cells in the culture maintained TLC-1 and were leucine independent (Table 19).

As a consequence of the replicational instability of TLC-1 even in RAD+ cells carrying Scp1, strains which are chromosomally leu2 can1 and carry the plasmid appear to be canavanine resistant when they are plated on medium that contains both leucine and canavanine. Single colonies of MSW28-10C transformed with TLC-1 were inoculated into 2.0 ml aliquots of liquid SC-leucine. After 48 hours growth at 30° C, suitable dilutions were spread on SC-leucine, SC, and SC + canavanine plates. Approximately 70% of the cells grown under these conditions contain plasmid TLC-1; approximately

TABLE 18

Mitotic stability of plasmid TLC-1 in RAD+ cir° strains grown in liquid YEPD

Strain	Culture	Number of Colonies		%LEU2
		LEU2	leu2	
YT6-2-1L[TLC-1]	1	0	137	0
	2	0	201	0
	3	0	164	0
	4	0	189	0
	5	0	197	0

TABLE 19

Mitotic stability of plasmid TLC-1 in *RAD*⁺ *cir*⁰ strains grown in liquid SC-leucine

Strain	Culture	Number of Colonies SC[1]	Number of Colonies SC-leu[2]	%LEU2
YT6-2-1L[TLC-1]	1	232 214	157 143	6.7
	2	264 234	101 *	4.1
	3	66 86	14 16	2.0
	4	256 273	149 166	6.0

[1] Dilution factor 0.2×10^{-4} for Synthetic Complete

[2] Dilution factor 0.2×10^{-3} for Synthetic Complete minus leucine

* Not determined

50% of the total cells are capable of forming colonies in the presence of canavanine when the plates contain leucine. Virtually all the canavanine resistant colonies which appear result from loss of TLC-1 and become leucine requiring (Table 20).

Recombinational Instability of Plasmid TLC-1

When strains which are chromosomally leu2 can1 and which carry TLC-1 are plated on medium containing canavanine but lacking leucine, the number of cells that are capable of growth is reduced compared to similar strains lacking the plasmid. When MSW28-10C or GRF18 cells transformed with TLC-1 are grown in liquid SC-leucine medium, only about 1 in 1,000 cells in the culture are capable of growth on medium containing canavanine but lacking leucine. This establishes that TLC-1 confers canavanine sensitivity to canavanine resistant cells which carry it, as would be expected from the dominance of the sensitivity over the resistance (Table 21). However, it is necessary to force the maintenance of TLC-1 by selecting for the LEU2 marker.

Although strains carrying plasmid TLC-1 are canavanine sensitive when grown under conditions that require maintenance of the plasmid, the frequency at which canavanine resistant, leucine independent cells arise is high, about 1 in 1,000. The process by which these cells arise requires the wild type RAD52 gene. When cultures of

TABLE 20

Viability of cells of RAD+ cir+ strain MSW28-10C[TLC-1] in the presence of canavanine and the presence of leucine

Culture	Number of Colonies SC'	SC-leu'	SC+can'	%Cells LEU2	can1
1	561	413	291	74	51
	563	418	281		
2	576	361	282	67	50
	557	403	279		
3	662	423	265	68	44
	591	428	282		
4	540	378	281	66	49
	590	368	269		
5	667	390	278	62	42
	596	399	259		

' Dilution factor 0.2 x 10^{-4} for all three media:
Synthetic Complete
Synthetic Complete minus leucine
Synthetic Complete plus canavanine

TABLE 21

Viability of RAD$^+$ cir$^+$ strains containing TLC-1 in the presence of canavanine and the absence of leucine

Strain	Culture	Number of Colonies SC-leu[1]	Number of Colonies SC-leu+can[2]	Frequency of LEU2 can1 ($\times 10^4$)
MSW28-10C [TLC-1]	1	189 226	53 87	3.4
	2	241 206	351 322	15.0
	3	228 258	229 240	9.6
	4	216 230	675 779	33.0
	5	188 211	134 180	8.1
GRF18 [TLC-1]	1	346 –	272 297	8.2
	2	400 –	318 299	7.7
	3	427 –	420 421	9.8
	4	139 –	1577 1562	110.0
	5	403 –	346 369	8.9

[1] Dilution factor 0.1×10^{-4} for Synthetic complete minus leucine

[2] Dilution factor 0.1×10^{-1} for Synthetic complete minus leucine plus canavanine

MSW152-1A [TLC-1] are grown for 48 hours in liquid SC-leucine medium and plated on SC-leucine plates containing canavanine, less than 1 in 100,000 cells are capable of forming a colony (Table 22).

The reduced frequency of production of canavanine resistant, leucine independent cells segregates with the rad52-1 mutation. Strain MSW152-1A [TLC-1] was crossed to strain MSW28-10C to generate diploid D155, which was then sporulated. Tetrads in which all four spores formed colonies that were unstably leucine independent were selected; the RAD52 spores produced cultures with high frequencies of LEU2 can1 cells, the rad52-1 spores produced cultures with low frequencies of canavanine resistant, leucine independent cells (Table 23).

The formation of LEU2 can1 colonies is also reduced in strains that lack Scp1. Strains GRF18 (cir+) and YT6-2-1L (cir°) carrying TLC-1 were grown for 48 hours under conditions requiring maintenance of TLC-1 and then spread on SC-leucine plates containing canavanine. Approximately 1 in 1,000 cells of strain GRF18 [TLC-1] were capable of growth on these plates (Table 21); less than 2 in 100,000 cells of YT6-2-1L [TLC-1] could grow (Table 24).

TABLE 22

Viability of cells of rad52-1 cir° strain containing TLC-1 in the presence of canavanine and the absence of leucine

Strain	Culture	Number of Colonies SC-leu[1]	SC-leu+can[2]	Frequency of $\frac{LEU2}{can1}$ (x 10^4)
MSW28-10C [TLC-1]	1	140	15	8.8
		145	10	
	2	140	2	1.8
		141	3	
	3	142	8	7.0
		143	12	
	4	143	12	6.6
		144	7	
	5	123	6	6.6
		118	10	

[1] Dilution factor 0.1 x 10^{-4} for Synthetic complete minus leucine

[2] Dilution factor 0.1 for Synthetic complete minus leucine plus canavanine

TABLE 23

Segregation of *rad52-1* and low frequency of *LEU2 can1* colony formation in cross MSW152-1A[TLC-1] x MSW28-10C

Spore	RAD52	Frequency of *LEU2 can1* ($\times 10^5$)
1A	-	1.4
1B	+	>5000.0
1C	-	0.6
1D	+	15.0
2A	-	3.0
2B	+	70.0
2C	-	3.0
2D	+	160.0

TABLE 24

Viability of RAD+ cir° strain containing TLC-1 in the presence of canavanine and the absence of leucine

Strain	Culture	Number of Colonies SC-leu[1]	SC-leu+can[2]	Frequency of LEU2 can1 (x 10^{5})
YT6-2-1L [TLC-1]	1	157	4	4.0
		143	8	
	2	101	62	61.0
		—	61	
	3	14	0	<3.3
		16	0	
	4	149	2	0.95
		166	1	

[1] Dilution factor 0.2 x 10^{-3} for Synthetic complete minus leucine

[2] Dilution factor 0.2 for Synthetic complete minus leucine plus canavanine

Characteristics of the Recombinational Instability of Plasmid TLC-1

The canavanine resistant, leucine independent colonies that arise in the RAD52 strains containing Scp1 (RAD+) cir+ contain a mitotically unstable LEU2 marker. Although the level of instability is high, the canavanine resistant, leucine independent derivatives of MSW28-10C [TLC-1] are more mitotically stable for the LEU2 marker than the original MSW28-10C [TLC-1] transformant. Single colonies of MSW28-10 [TLC-1] and each of the five independent LEU2 can1 derivatives were purified on SC-leucine plates and were inoculated into 2.0 ml aliquots of liquid YEPD. The cultures were assayed for leucine requiring cells at two times; after growth to saturation of the original inoculum, and after growth to saturation of a 1 to 10,000 dilution of a previously saturated culture. At both times the culture of the canavanine resistant derivatives contained a higher proportion of leucine independent cells than did the cultures of MSW28-10C [TLC-1] (Table 25).

The LEU2 can1 derivatives of MSW28-10C [TLC-1] do not contain plasmid TLC-1 although the LEU2 marker is still found on a plasmid. Bulk DNA was isolated from MSW28-10C [TLC-1] and from each of the five independent LEU2 can1 derivatives. The unrestricted DNA was analyzed by Southern blotting using nick translated plasmid p36-1B as a probe. This probe is specific for unique sequences at the yeast

TABLE 25

Mitotic stability of *LEU2* marker in strain MSW28-10C[TLC-1] and in *LEU2* *can1* derivatives of strain MSW28-10C[TLC-1]

	GROWTH TO SATURATION					
	Original Inoculum			Diluted Saturated Culture		
Strain	Number of Colonies *LEU2*	*leu2*	%*LEU2*	Number of Colonies *LEU2*	*leu2*	%*LEU2*
MSW28-10C[TLC-1]	983	874	47	25	98	20
LEU2 *can1* #28-1	744	177	81	122	54	69
LEU2 *can1* #28-2	814	129	86	127	54	70
LEU2 *can1* #28-3	805	106	88	147	38	79
LEU2 *can1* #28-4	691	65	91	152	38	80
LEU2 *can1* #28-5	386	23	94	154	56	73

LEU2 locus; it hybridized to both high molecular weight chromosomal DNA and low molecular weight plasmid DNA in all six strains. However, the plasmid with homology to plasmid p36-1B in strain MSW28-10C [TLC-1] was larger than the plasmid in the LEU2 can1 derivatives. All five independent LEU2 can1 derivatives carried a similar LEU2 plasmid (Figure 11B). The LEU2 can1 derivatives of MSW28-10C [TLC-1] do not contain sequences homologous to plasmid pBR322. Southern analysis using nick translated pBR322 as a probe was done on the unrestricted DNA of MSW28-10C [TLC-1] and the five LEU2 can1 derivatives. Only the DNA from MSW28-10C [TLC-1] contained homology to the probe (Figure 11A).

There were no biologically detectable plasmids containing pBR322 in the LEU2 can1 derivatives. The bulk DNA from MSW28-10C [TLC-1] and the five LEU2 can1 derivatives were used to transform E. coli strain JF1754 to ampicillin resistance. Greater than 300 ampicillin resistant transformants were obtained per microgram of DNA from MSW28-10C [TLC-1], and 50 out of 50 transformants tested were also leucine independent. No ampR leuB transformants were obtained from the LEU2 can1 derivatives (Table 26).

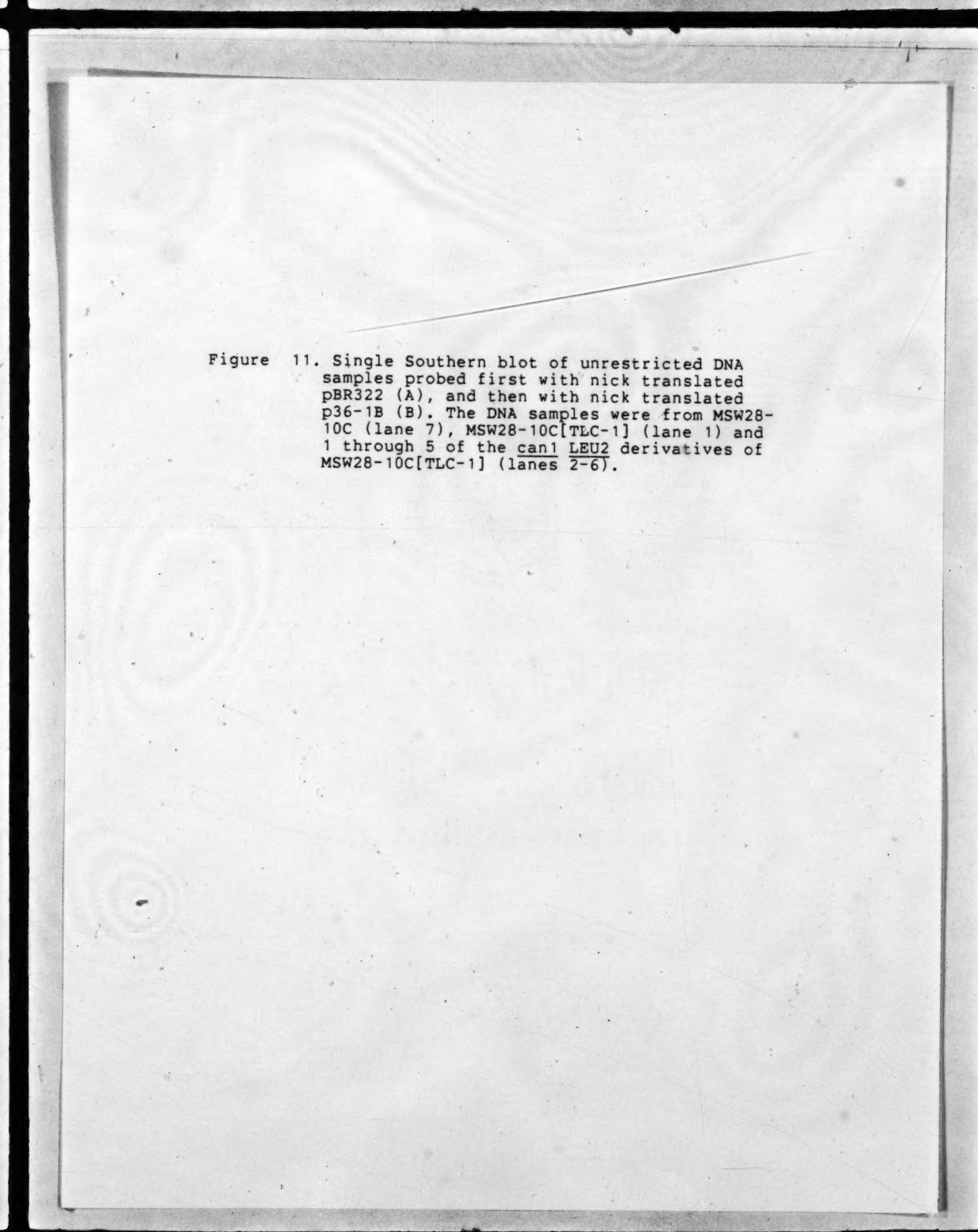


Figure 11. Single Southern blot of unrestricted DNA samples probed first with nick translated pBR322 (A), and then with nick translated p36-1B (B). The DNA samples were from MSW28-10C (lane 7), MSW28-10C[TLC-1] (lane 1) and 1 through 5 of the *can1* *LEU2* derivatives of MSW28-10C[TLC-1] (lanes 2-6).

A

1 2 3 4 5 6 7

B

1 2 3 4 5 6 7

TABLE 26

Transformation of *E. coli* with DNA isolated from *RAD*⁺ yeast strains

Source of DNA	Micrograms DNA	Number of Transformants	
		Amp R[1]	*LeuB*[2]
MSW28-10C[TLC-1]	1	332	50/50
LEU2 can1 #28-1	1	0	–
LEU2 can1 #28-2	1	0	–
LEU2 can1 #28-3	1	0	–
LEU2 can1 #28-4	1	0	–
LEU2 can1 #28-5	1	1	0/1

[1] Ampicillin resistant

[2] Number of *LeuB* colonies out of number tested

Effect of rad52-1 on Recombination Instability of TLC-1

The canavanine resistant, leucine independent colonies that arise in the rad52-1 cir+ background contain mitotically unstable LEU2 markers. Five independent LEU2 can1 derivatives of MSW152-1A [TLC-1] were isolated. These five derivatives fell into two classes on the basis of the behaviour of the plasmids they carried; derivatives 1, 3 and 5 behaved similarly to strain MSW152-1A [TLC-1], derivatives 2 and 4 behaved like the LEU2 can1 derivatives of MSW28-10C [TLC-1].

The mitotic stability of the LEU2 marker in derivatives 1, 3 and 5 was similar to the mitotic stability of TLC-1 in MSW152-1A; derivatives 2 and 4 had a more stable LEU2 marker. Single colonies from SC-leucine plates were inoculated into 2.0 ml aliquots of liquid YEPD and grown at 30°C for 24 hours. Suitable dilutions were spread on YEPD plates, then replica plated to SC-leucine medium to estimate the proportion of cells in each culture that had lost the LEU2 marker. Strain MSW152-1A [TLC-1] and derivatives 1, 3 and 5 had about 40% of the cells still carrying the LEU2 marker, while derivatives 2 and 4 had approximately 60% of the cells still LEU2 (Table 27).

Strain MSW152-1A [TLC-1] and derivatives 1, 3 and 5 contain physically detectable pBR322 sequences; derivatives 2 and 4 do not. Bulk DNA was isolated from MSW152-1A [TLC-1]

TABLE 27

Mitotic stability of *LEU2* marker in strain MSW152-1A[TLC-1] and in *LEU2* *can1* derivatives of strain MSW152-1A[TLC-1]

Strain	Number of Colonies		%*LEU2*
	LEU2	*leu2*	
MSW152-1A[TLC-1]	94	168	35
LEU2 *can1* #152-1	112	175	39
LEU2 *can1* #152-2	143	107	57
LEU2 *can1* #152-3	172	243	41
LEU2 *can1* #152-4	168	104	62
LEU2 *can1* #152-5	106	159	40

and the five LEU2 can1 derivatives, fractionated on a 1% agarose gel, and blotted to a nitrocellulose filter. This filter was hybridized with nick translated pBR322. Only derivatives 1, 3 and 5, together with MSW152-1A [TLC-1], contained detectable homology to pBR322 (Figure 12A).

When the filter is hybridized with nick translated p36-1B, homology is found in all five derivatives as well as in strain MSW152-1A[TLC-1]. The LEU2 plasmid in derivatives 2 and 4 is smaller than the LEU2 plasmid found in MSW152-1A[TLC-1] and derivatives 1, 3 and 5 (Figure 12B).

Strain MSW152-1A[TLC-1] and derivatives 1, 3 and 5 carry biologically detectable plasmids containing pBR322; derivatives 2 and 4 do not. Bulk DNA from MSW152-1A [TLC-1] and the five LEU2 can1 derivatives was used to transform E. coli strain JF1754 to ampicillin resistance. Ampicillin resistant E. coli colonies that were also leuB$^+$ were obtained from DNA isolated from strain MSW152-1A [TLC-1] and derivatives 1, 3 and 5, but no ampicillin resistant colonies were obtained from the DNA of derivatives 2 and 4 (Table 28).

The ampR leu$^+$ E. coli transformants obtained from the DNA from MSW152-1A [TLC-1] and the LEU2 can1 derivatives 1, 3 and 5 contained plasmids identical in size to TLC-1. A single ampR leu$^+$ transformant of JF1754 obtained from each yeast DNA preparation was analyzed by rapid plasmid DNA

Figure 12. Southern blot of unrestricted DNA samples probed first with nick- translated pBR322 (A) and then with nick-translated p36-1B (B). The DNA samples were from MSW28-10C[TLC-1] (lanes 1 and 8), MSW152-1A[TLC-1] (lane 2) and 1 through 5 of the $\frac{can1}{can1}$ $\frac{LEU2}{LEU2}$ derivatives of MSW152-1A[TLC-1] (lanes 3-7).

A

B

TABLE 28

Transformation of *E. coli* with DNA isolated from *rad52-1* yeast strains

Source of DNA	Micrograms DNA	Number of Transformants	
		Amp R[1]	*LeuB*[2]
MSW152-1A[TLC-1]	1	7	7/7
LEU2 can1 #152-1	1	22	10/10
LEU2 can1 #152-2	1	0	–
LEU2 can1 #152-3	1	73	10/10
LEU2 can1 #152-4	1	0	–
LEU2 can1 #152-5	1	2	2/2

[1] Ampicillin resistant

[2] Number of *LeuB* colonies out of number tested

isolation. The ampicillin resistant leucine independent transformants from the DNA of MSW152-1A [TLC-1] and the transformants from the DNA of derivatives 1, 3 and 5 all contained plasmids that co-migrated on 1% agarose gels with purified TLC-1 DNA.

Effect of Scp1 on Recombinational Instability of TLC-1

The LEU2 can1 derivatives of RAD+ cir° strain YT6-2-1L [TLC-1] do not contain mitotically unstable LEU2 markers. Three independent LEU2 can1 derivatives of YT6-2-1L [TLC-1] were purified on SC-leucine plates and single colonies inoculated into liquid YEPD. The cultures were grown for 24 hours at 30°C, and suitable dilutions were plated on YEPD plates. After 2 days growth at 30°C the colonies were replica plated to SC-leucine plates. Less than 0.5% of the cells in these cultures were leucine requiring; the LEU2 can1 colonies that appeared in the cir° strain contained a mitotically stable LEU2 marker (Table 29).

The data presented so far establish that the production of LEU2 can1 cells at a high frequency in cultures of leu2 can1 yeast strains carrying plasmid TLC-1 involves the formation of a LEU2 plasmid which lacks detectable homology to pBR322 and is smaller than TLC-1. They also show that the formation of this new LEU2 plasmid involves the wild type allele of the RAD52-1 gene, and also requires the endogenous yeast plasmid Scp1.

TABLE 29

Mitotic stability of LEU2 marker in strain YT6-2-1L[TLC-1] and in LEU2 can1 derivatives of strain YT6-2-1L[TLC-1]

Strain	Number of Colonies		%LEU2
	LEU2	leu2	
YT6-2-1L[TLC-1]	0	280	0.0
LEU2 can1 #T6-1	311	0	100.0
LEU2 can1 #T6-2	361	1	>99.7
LEU2 can1 #T6-3	330	1	>99.7

Interpretation of Recombinational Instability of TLC-1

A model which accommodates these observations is that recombination between Scp1 and TLC-1 separates the LEU2 region of TLC-1 from the pBR322 CAN1 segment. The LEU2 region of TLC-1 is flanked by sequences from Scp1; recombination between TLC-1 and Scp1 at these regions of homology can generate a new plasmid by exchanging the PstI fragment of TLC-1 containing the LEU2 region. This plasmid consists of Scp1 with the LEU2 fragment inserted at the PstI site. This LEU2 Scp1 plasmid will be maintained in cells grown on SC-leucine + canavanine plates; plasmids carrying CAN1 will be lost.

This model predicts that the LEU2 plasmid found in the can1 LEU2 derivatives of MSW28-10C [TLC-1] would be 10.7 kb, the sum of Scp1 and the PstI fragment of TLC-1 containing the LEU2 region. The DNA was isolated from MSW28-10C [TLC-1] and the five can1 LEU2 derivatives. This DNA was fractionated, together with purified TLC-1 and a multimeric series of pBR322 molecules as molecular weight standards, on a 1% agarose gel. The fractionated DNA was blotted to nitrocellulose, and the filter was probed with nick translated p36-1B. The plasmid in the can1 LEU2 derivatives migrated between the dimeric and trimeric forms of pBR322, at an estimated molecular weight of 10.7 kb. This is in good agreement with the expected size of a Scp1-LEU2 plasmid. Purified TLC-1 migrated between the trimeric and tetrameric

forms of pBR322, at an estimated molecular weight of 15.0 kb (Figure 13).

This model also predicts that the LEU2 plasmid in the can1 LEU2 derivatives of MSW28-10C [TLC-1] contains sequences from Scp1 not found in TLC-1. A probe specific for sequences of Scp1 not found in TLC-1 was generated by purifiying the 1.3 kb HindIII fragment of pTM2 by preparative gel electrophoresis. This fragment encompasses coordinates 1017 to 2331 of the A form of Scp1 (Hartley and Donelson, 1980); TLC-1 contains sequences from coordinates 0 to 939 and 2407 to 3714.

The can1 LEU2 derivatives of MSW28-10C [TLC-1] contain low molecular weight DNA species, not found in MSW28-10C [TLC-1], that have homology to the Scp1 probe. The probe for specific sequences of Scp1 not found in TLC-1 was hybridized to fractionated unrestricted DNA from MSW28-10 [TLC-1] and the five can1 LEU2 derivatives. All five can1 LEU2 derivatives contained identical low molecular weight species, not found in MSW28-10C [TLC-1], that hybridized to the probe. In addition, MSW28-10C [TLC-1] and can1 LEU2 derivatives 1 and 2 contained low molecular weight DNA species in common; these presumably represent different forms of the endogenous yeast plasmid Scp1 (Figure 14A).

The can1 LEU2 derivatives of MSW28-10C [TLC-1] carry plasmids that contain both Scp1 sequences not found in TLC-1

Figure 13. Southern blot of unrestricted DNA samples probed with nick translated p36-1B. The DNA samples were a multimeric series of pBR322 (lane 1), bulk DNA from MSW28-10C[TLC-1] (lane 2) and 1 through 5 of the can1 LEU2 derivatives of MSW28-10C[TLC-1] (lanes 3-7), and TLC-1 (lane 8). The sizes in kilobases of the various forms of pBR322 are indicated. pLEU2 represents the novel LEU2 plasmid found in can1 LEU2 derivatives of MSW28-10C[TLC-1].

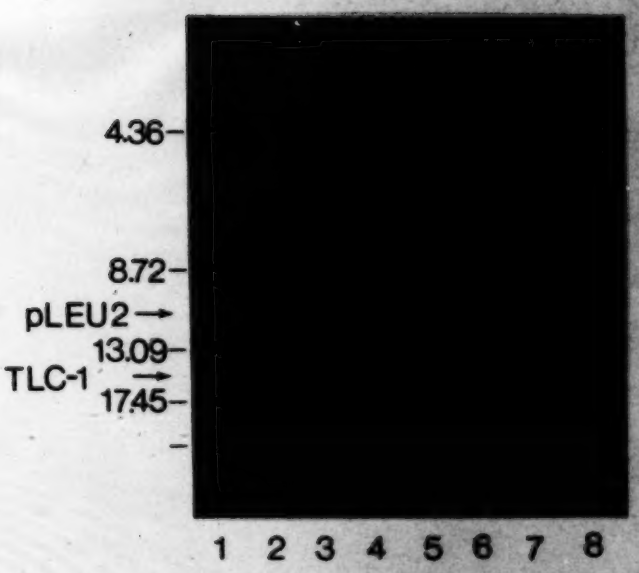
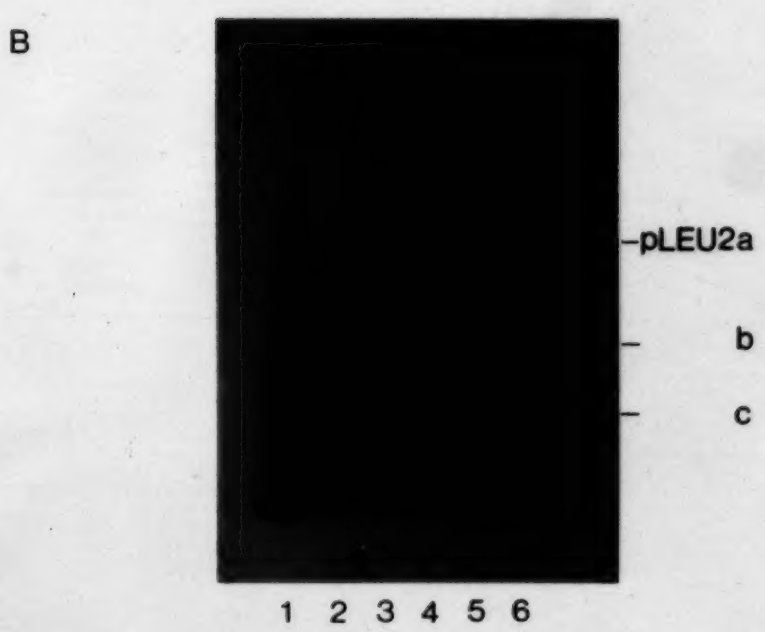

4.36
8.72
pLEU2
13.09
TLC-1
17.45
1 2 3 4 5 6 7 8

Figure 14. Southern blot of unrestricted DNA samples probed first with a nick translated probe containing nucleotides 1017 to 2331 of the A form of Scp1 (A), and then reprobed, after the original signal had decayed, with nick translated p36-1B (B).The DNA samples were bulk DNA from MSW28-10C[TLC-1] (lane 1) and 1 through 5 of the can1 LEU2 derivatives of MSW28-10C[TLC-1] (lanes 2-6). The DNA species corresponding to a, b and c are various forms (supercoiled, linear and open circular) of Scp1 and pLEU2 respectively. Supercoiled pLEU2 and linear Scp1 run to the same position.

A
Scp1 a–
b–
c–
–pLEU2a
– b
– c
1 2 3 4 5 6


B
–pLEU2a
– b
– c
1 2 3 4 5 6

and sequences from the LEU2 region. A single nitrocellulose filter containing fractionated DNA from MSW28-10C [TLC-1] and the five can1 LEU2 derivatives was probed twice; first with the probe specific for Scp1 sequences not found in TLC-1, and then with the LEU2 pBR322 probe p36-1B. The low molecular weight DNA species with homology to the Scp1 probe that were found only in the can1 LEU2 derivatives also hybridized the LEU2 probe. The low molecular weight species with homology to the Scp1 probe that were found in MSW28-10C [TLC-1] and can1 LEU2 derivatives 1 and 2 did not hybridize the LEU2 probe (Figure 14 A&B).

DISCUSSION

Mitotic recombination in S. cerevisiae has classically been studied by investigating exchanges between heterozygous regions of homologous chromosomes in diploid cells. However, recombination is not confined to diploids, and can occur between duplicated sequences in haploid cells. Mating type switching (Klar et al., 1980; Haber et al., 1980), and intramolecular reciprocal recombination between the inverted repeats of Scp1 (Broach et al., 1982) are examples of such recombinational processes occurring in haploid cells. Both mating type switching and Scp1 intramolecular recombination occur at high frequencies and are under the control of gene products specific for the particular recombination system.

Developments in recombinant DNA technology have allowed for the isolation of yeast genes and their propagation in E. coli (Struhl et al., 1976; Ratzkin and Carbon, 1977), and for the reintroduction of these genes into yeast cells (Hinnen et al., 1978). Artificial duplications of yeast chromosomal sequences have been constructed which allow the detection of generalized mitotic recombination in haploid cells (Scherer and Davis, 1980; Szostak and Wu, 1980; Jackson and Fink, 1981). Mitotic recombination in diploids can be monitored by the formation of prototrophic cells from diploid strains carrying heteroalleles which confer an auxotrophic requirement (Roman, 1956; Roman and Jacob, 1958); analogous systems have been developed for detecting

recombination in haploid cells (Scherer and Davis, 1980; Jackson and Fink, 1981). Mitotic recombination can also be detected in diploid cells heterozygous for a recessive allele for drug resistance or colony colour by observing cells expressing the mutant trait (Roman, 1956). The present work extends this latter approach to the investigation of mitotic recombination in haploid cells. Mitotic recombination is detected by the uncovering of the recessive resistance to the arginine analogue canavanine in haploid strains that carry both a mutant and wild type copy of the CAN1 gene. This system has been used to detect recombination occurring between repeated sequences in the yeast chromosome, and between repeated sequences occurring on autonomously replicating plasmids.

RECOMBINATION BETWEEN CHROMOSOMAL REPEATS

The development of a system that would allow genetic detection of recombination between duplicated sequences on the chromosomes of haploid yeast cells involved the construction of a haploid strain of S. cerevisiae with both a chromosomal wild type and a chromosomal mutant allele of the CAN1 gene. This manipulation required the insertion of an in vitro generated mutant can1 sequence into a strain with a wild type CAN1 locus. Plasmid p47-3 was made by deleting from plasmid TLC-1 both the Scp1 origin of replication and a 1.2 kb sequence from within the 4.5 kb BamHI fragment containing the CAN1 gene. Removal of the 1.2 kb HindIII fragment inactivates the CAN1 gene, so a leu2 can1 strain (GRF18) remains canavanine resistant when transformed to leucine independence with p47-3. The removal of the Scp1 origin of replication reduces the transformation efficiency of the plasmid. Three orders of magnitude more LEU2 transformants per microgram DNA are generated by TLC-1 than are generated by p47-3.

Because the removal of the Scp1 origin of replication prevents autonomous replication of p47-3 in yeast, the formation of LEU2 transformants requires that p47-3 integrate into a piece of DNA capable of replication. Transformation of a leu2 strain with p47-3 generates, in addition to true LEU2 transformants, a large number of tiny leucine "independent" colonies that are leu2 when

restreaked. These colonies are probably the result of cells that obtained one or more copies of p47-3 during transformation and were able to undergo enough cell divisions in the absence of leucine to form a small colony, but which failed to integrate p47-3 into DNA sequences capable of replication. Therefore, these colonies did not become stably LEU2 .

Structure of Transformants Containing p47-3

Plasmid p47-3 was used to transform strain MSW5-21A, (which was CAN1 and contained two point mutations in the LEU2 gene), to leucine independence. Five independent transformants had the LEU2 marker of plasmid p47-3 integrated at five different locations. Four of the LEU2 transformants had mitotic and meiotic segregation patterns characteristic of a chromosomal location of the LEU2 marker; the marker was very stably maintained through mitosis, and generally segregated as a single Mendelian gene during meiosis. One transformant was mitotically unstable for the LEU2 phenotype, and its LEU2 marker segregated as a cytoplasmic element during meiosis. This transformant resulted from p47-3 integrating into a copy of the endogenous yeast plasmid Scp1.

Integration of Scp1 into p47-3 occurred by a single crossover between the two molecules within an identical 245 base pair sequence found on both plasmids. Therefore, DNA

sequences incapable of autonomous replication must come in contact with the endogenous yeast plasmid during the transformation process, and reciprocal recombination can occur between regions of Scp1 that are not part of the inverted repeat. It is likely that this recombination event is independent of the specialized Scp1 recombination system, because recombination mediated by the FLP gene is restricted to a specific region of the inverted repeat sequence (Broach et al., 1982). The observation that p47-3 and Scp1 can recombine suggests that generalized reciprocal recombination can occur between DNA molecules that contain as little as 245 base pairs of homology.

One out of five transformants had p47-3 integrated into Scp1, even though the fragment of p47-3 with homology to Scp1 was considerably smaller than the regions of p47-3 with homology to chromosomal sequences. This is probably a consequence of the copy number of the yeast plasmid, which is found at about 50 molecules per cell (Clarke-Walker and Miklos, 1974; Gerbaud and Guerineau, 1980). Therefore, although there are extensive chromosomal sequences with homology to p47-3, there are a large number of copies of the small Scp1 sequence with p47-3 homology, and integration into both the yeast chromosome and into Scp1 can be detected.

One of the four chromosomal transformants had p47-3 integrated at the CAN1 locus. This location was established

by meiotic mapping studies which showed that the LEU2 marker of p47-3 was tightly linked to the CAN1 locus, and by Southern analysis that showed the integration of p47-3 had disrupted the normal pattern of EcoRI cleavage sites within the CAN1 region. The other three chromosomal transformants had integrated p47-3 at three other locations. Meiotic mapping was used to show that these three transformants had the LEU2 marker of p47-3 inserted at sites unlinked to either the normal LEU2 locus or to the CAN1 locus. In addition, mapping studies showed that the three LEU2 transformants contained the LEU2 marker at three separate sites unlinked to one another.

Other workers have detected chromosomal integration of plasmids carrying the LEU2 region isolated from S288C derived yeast strains at locations distinct from the LEU2 locus, (Hicks et al., 1978; Hinnen et al., 1978). The LEU2 region contains sequences that are repeated at many locations in the yeast genome. The LEU2 region from S288C and its derivatives contains a yeast transposable element Ty-17 and its associated delta elements, and an RNA gene that is repeated at other chromosomal locations (Dobson et al., 1981). Integration of p47-3 at sites unlinked to either CAN1 or LEU2 is presumably the result of crossing-over between a repeated sequence found on p47-3 and a similar sequence in the yeast genome (Hicks et al., 1978).

Two of the three chromosomal integrants of p47-3 that

had presumably recombined at a repeated sequence were meiotically unstable. When crossed to strain Y0555-19A, one exhibited aberrant segregation of the LEU2 marker of p47-3 in 2 of 10 complete tetrads analyzed; the other exhibited aberrant segregation in 2 of 14 complete tetrads. The transformant with p47-3 integrated at the CAN1 locus, and one of the transformants with p47-3 integrated at a repeated sequence were more stable, since no aberrant LEU2 segregations were detected in 43 complete tetrads analyzed. It has been noted that transformants containing chromosomal duplications of the LEU2 region are meiotically unstable (Klein and Petes, 1981); the differing meiotic stabilities of the p47-3 transformants may reflect differences in the chromosomal structure of the various integrants. However, no significant differences in the mitotic stabilities of the LEU2 markers were noted.

Mitotic Instability of Transformants

The transformant that contained p47-3 integrated at the CAN1 locus was mitotically unstable for the CAN1 and LEU2 phenotypes. The untransformed strain MSW5-21A segregated can1 derivatives at a frequency of about 5.5×10^{-7} can1 per cell; the derivatives of MSW5-21A carrying p47-3 integrated at the CAN1 locus segregated can1 cells at a frequency of about 2.2×10^{-4} can1 per cell. About 4% of the can1 derivatives of MSW5-21A with p47-3 integrated at the CAN1

locus were also leu2. Transformants containing plasmid p47-3 integrated at sites unlinked to CAN1 were more stable, and did not generate can1 cells at frequencies significantly different from the untransformed strain.

The instability of the CAN1 locus of MSW5-21A containing p47-3 is chiefly due to mitotic rearrangements, most of which require the RAD52 gene product. These will be discussed below. Southern analyses were performed on 22 independent canavanine resistant derivatives and 11 independent leucine requiring derivatives obtained in a RAD52 background; all but one of the canavanine resistant derivatives, and all of the leucine requiring derivatives, had undergone structural rearrangements at the CAN1 locus. Structural changes were less common in strains containing the rad52-1 allele, as only 1 of 9 independently isolated canavanine resistant derivatives obtained in a rad52-1 background had undergone mitotic rearrangement.

Mutation

One of the 22 can1 derivatives of MSW5-21A containing p47-3 integrated at the CAN1 locus did not contain a structural rearrangement, and apparently arose through a point mutation in the CAN1 gene. The frequency of mutation was $1/22 \times 2.2 \times 10^{-8}$, or 1×10^{-9} can1 mutations per cell. This is similar to the frequency of mutation of the wild type CAN1 gene in RAD52 cells. This result confirms that haploid

strains carrying the duplicated CAN1 locus are heterozygous for the functional CAN1 allele; if both CAN1 sequences encoded a functional permease, the formation of can1 mutants would occur at a considerably lower frequency.

Point mutants of the functional CAN1 gene occurred in 8 of the 9 can1 derivatives of the rad52-1 strain containing p47-3 integrated at the CAN1 gene. The frequency of formation of can1 cells in rad52-1 strains containing the CAN1 duplications is the same as for RAD52 strains, so the mutational frequency is 8/9 x 2.2×10^{-8}, or about 2×10^{-8} can1 per cell. This is a 20 fold enhancement over the mutational frequency in RAD52 cells containing the CAN1 duplication. Other researchers have noted that the rad52-1 allele increases the spontaneous mutation frequency at a variety of loci (von Borstel et al., 1971; Prakash et al., 1980).

Structural Rearrangements

The remaining derivatives obtained in the RAD52 background contain structural rearrangements that fall into two classes: intrachromosomal gene conversion, and events that resolve a duplication by removing one copy of the direct repeat and intervening DNA between the two repeats.

Gene Conversion

Four intrachromosomal gene conversions were detected in 21 independent can1 LEU2 derivatives of strain MSW5-21A/p47-3 #1. These events had replaced the functional CAN1 sequence within the 5 kb EcoRI fragment with the non-functional copy of the CAN1 sequence on the 14 kb EcoRI fragment. The copy on the 14 kb fragment has an internal deletion of 1.2 kb, therefore the mitotic conversion event replaces the wild type CAN1 gene with a mutant allele containing a deletion of the CAN1 information. Previously, conversion of deletions in S. cerevisiae has been detected genetically during meiosis (Fink and Styles, 1974; Lawrence et al., 1975; Fogel et al., 1978), and physically during mitosis (Scherer and Davis, 1980). In addition, conversion of large regions of heterozygosity presumably occur during mating type switching in homothallic (HO) and heterothallic (ho) strains (Klar et al., 1980; Haber and Rogers, 1982). The meiotic studies suggest that conversions of deletions may, in some aspects, be similar to conversions of point mutations. At least during meiosis, large regions of non-homology do not appear to significantly perturb the conversion process.

The formation of can1 LEU2 cells by conversion of the wild type sequence of the duplicated CAN1 locus to the deleted sequence is reduced in strains that carry the rad52-1 mutation. Four of 22 can1 derivatives obtained in a RAD52 background were conversion events and so arose at an

approximate frequency of 4/22 x 2.2×10^{-5} or 4×10^{-6} conversion events per cell. No conversion events were detected in 9 can1 derivatives obtained from a strain containing both the CAN1 duplication and the rad52-1 allele. The frequency of conversion events in these strains is less than 1/9 x 2.2×10^{-5}, or 2.5×10^{-6} conversion events per cell. Although the reduction is not large, it is significant that no conversions were detected in the rad52-1 strain. Similar results have been obtained for intrachromosomal conversions between duplicated HIS4 gene sequences in the presence of the rad52-1 mutation (Jackson and Fink, 1981).

Resolution of Non-contiguous Direct Repeats

Seventeen of 21 can1 LEU2 derivatives and 11 of 11 CAN1 leu2 derivatives of strain MSW5-21A/p47-3#1 arose through resolution of non-contiguous direct repeats. These repeats can be diagrammatically represented as 12-X-12, where 12 represents the duplicated sequence, and X represents unique sequence between the duplicated elements. Resolution of the duplication results in the loss of one copy of the 12 information along with the X information, and leaves a single copy of the 12 sequence.

Previous studies in S. cerevisiae have attributed the resolution of such structures to reciprocal recombination. This recombination can occur either within the same DNA duplex to generate a circular excision product, or between

sister chromatids to delete the sequence from one chromatid and insert it in the other (Szostak and Wu, 1980; Zamb and Petes, 1981; Jackson and Fink, 1981). However, alternative processes can also resolve the duplications. The 12-X-12 structure can be considered as 12 with an insertion of 2-X-1 between 1 and 2; gene conversion using 12 as the template will resolve the duplication to the 12 sequence. Non-recombinational processes, such as replicational errors involving slippage of the template or replicating strand, could also result in deletion of the 2-X-1 sequence (Streisinger et al., 1966; Farabaugh et al., 1978).

A number of non-contiguous direct duplications were formed by integration of p47-3 at the CAN1 locus of MSW5-21A, and events which resolved each structure were detected. These events generate either CAN1 leu2, can1 LEU2, or jointly can1 leu2 derivatives of the orginally CAN1 LEU2 strain (see Figure 8). Two separate events can lead to the formation of CAN1 leu2 derivatives; one involves a duplication sequence of about 2.75 kb separated by approximately 7.5 kb (b1/b2); the other involves a duplicated sequence of about 0.15 kb also separated by about 7.5 kb (a2/a3). Six of ten CAN1 leu2 derivatives arose by resolution of the b1/b2 duplication; four arose by resolution of the a2/a3 duplication. The duplicated sequence involved in the b1/b2 resolution is about 15 times larger than the duplicated sequence involved in the a2/a3

resolution, and the two duplicated sequences are separated by almost the same region of unique DNA, yet the frequencies of the two events are similar. This establishes that the frequency of resolution of these duplications is not proportional to the size of the duplicated sequence.

Reciprocal and Non-reciprocal Recombination

It is possible to estimate the contribution of both reciprocal (resolution of direct repeats) and non-reciprocal (gene conversion) recombination in the formation of rearrangements at the duplicated CAN1 locus in strain MSW5-21A/p47-3 #1. The 1.2 kb deletion divides the CAN1 sequence into three regions, the 0.325 kb c regions and 2.75 kb b regions found in both the wild type CAN1 and deleted can1 sequences, and the 1.2kb region found only in the wild type CAN1 gene (see Figure 8). Rearrangements that involve reciprocal recombination, with or without an associated gene conversion, generate either can1 leu2 cells due to an event involving the c1/c2 duplication, or CAN1 leu2 cells due to an event involving the b1/b2 duplication. Gene conversions of the wild type CAN1 sequence to the deleted can1 sequence, without an associated cross-over, form LEU2 can1 cells. The approximate frequency of gene conversions from the wild type to the mutant CAN1 allele was 4×10^{-6} conversions per cell. Thirteen of 292 can1 cells analyzed were also leu2, so the frequency of events involving the c1/c2 duplication was

13/292 X 2.2 X 10^{-5} or 1 X 10^{-4} reciprocal events involving the c1/c2 duplication per cell. One out of 11 leu2 derivatives analyzed involved the c1/c2 event, whereas 6 involved the b1/b2 event. The frequency of b1/b2 reciprocal recombination therefore was 6 times that of c1/c2 reciprocal recombination, or approximately 6 X 10^{-4} reciprocal events involving the b1/b2 duplication per cell. The duplicated sequence involved in the b1/b2 resolution is about 8.5 times larger than the sequence involved in the c1/c2 resolution. Therefore, in this situation the frequency of resolution of non-contiguous direct repeats is about proportional to the size of the duplicated sequence.

It appears that reciprocal and non-reciprocal events are equally important in the formation of physical rearrangements at the duplicated CAN1 locus. If half the conversion events generate two functional CAN1 genes, and therefore, are not detected as can1 LEU2 cells, the total conversion frequency would be $8x10^{-4}$ conversions per cell. The total frequency of reciprocal events would be $7x10^{-4}$ reciprocal events per cell, so the two frequencies are nearly equal.

Similar studies have been performed on artificially constructed duplications of the HIS4 gene which carry different point mutations, and on the naturally occurring duplications of the mating type region. In experiments with duplicated his4 heteroalleles, 12% to 25% of the events

which generate HIS4 recombinants involve a reciprocal exchange (Jackson and Fink, 1981). In that study no attempt was made to detect reciprocal events that were not associated with the formation of HIS4 recombinants. In heterothallic (ho) yeast strains, about 20% of MAT alpha to MAT a switches are associated with reciprocal recombination (Strathern et al., 1979). Taken together, these results suggest that spontaneous intrachromosomal recombination between duplicated sequences in haploid S. cerevisiae frequently involves reciprocal exchange. This is similar to intragenic recombination during meiosis (Fogel et al., 1978) and mitosis (Esposito and Wagstaff, 1981).

Factors Affecting Recombination

Current models of genetic recombination postulate that the initial steps in the recombination process involve the formation of a cross-strand exchange between DNA duplexes (Holliday, 1964; Meselson and Radding, 1975), and suggest that the associated reciprocal exchange is a consequence of an isomerization between the exchanged and unexchanged strands. If such models are correct, factors which influence the isomerization process will affect the probability of reciprocal exchange associated with the recombination event. These factors may include the enzymes involved in the recombination process, for there is less reciprocal exchange during mating type switching in homothallic strains (Haber

et al., 1980) than in heterothallic strains (Strathern et al., 1979). These factors may also include the structures of the recombining loci. An integrative plasmid cleaved within a site of homology to the yeast chromosome generates transformants primarily by reciprocal recombination while the same plasmid cleaved outside the region of homology generates transformants primarily by gene conversion (Orr-Weaver et al., 1981). There is a wide variety in the frequency of associated reciprocal exchange during mitotic conversion at different loci in diploid cells (Roman and Jacob, 1958; Esposito, 1978; Roman, 1980), but recombination between duplicated sequences in haploid cells at both the CAN1 and HIS4 (Jackson and Fink, 1981) frequently involve reciprocal exchange.

In addition to influencing the frequency of associated reciprocal exchanges, the structure of the recombining DNA sequences affects their overall frequency of recombination. Integration of plasmid p47-3 at the CAN1 locus increases the formation of can1 derivatives by 40 fold; integration of p47-3 at sites unlinked to the CAN1 locus causes little or no increase in the frequency of can1 derivatives. Similarly, translocation of HML alpha from its normal position on chromosome III to a location on chromosome XII reduces MAT a to MAT alpha switching in homothallic strains by 100 fold (Haber et al., 1981). These observations suggest that the locations of the recombining loci have an important bearing

on the frequency of recombination between the sequences.

The structure of the recombining sequences also affects the enzymatic requirements for the recombination process. Resolution of different non-contiguous direct duplications can either require the RAD52 gene product, or be RAD52 independent. Resolution of a direct repeat of approximately 150 nucleotides separated by 2.75 kb occurs in 16 of 22 can1 derivatives of a RAD52 strain containing p47-3 integrated at the CAN1 locus, at an approximate frequency of 16/22 x 2.2×10^{-5}, or 1.6×10^{-5} resolution events per cell. The same event was not detected in 9 can1 derivatives of a rad52-1 strain containing p47-3 integrated at the CAN1 locus, and therefore occurs at a frequency of less than 1/9 x 2.2×10^{-5} or less than 2.5×10^{-6} resolution events per cell. In contrast, resolution of a direct repeat of approximately 325 nucleotides separated by 8 kb occurs at similar frequencies in RAD52 and rad52-1 cells; the recombination event occurs in 13 of 292 can1 derivatives of a RAD52 strain, and 11 of 290 derivatives of a rad52-1 strain. Therefore it occurs at a frequency of about 1×10^{-6} events per cell in each strain. This shows that resolution of similar structures may have different requirements for the RAD52 gene product.

Previous experiments suggest that the rad52-1 mutation dramatically reduces non-reciprocal mitotic recombination, but has little or no effect on reciprocal mitotic recombination (Jackson and Fink, 1981). The observation that

resolution of a particular non-contiguous direct repeat is eliminated in a rad52-1 strain means either that the resolution of this structure occurs not by reciprocal recombination, but by conversion or replication error, or that some reciprocal events do require the RAD52 gene product. Because similar structures can be resolved independently of the rad52-1 mutation, it appears there is a sequence or distance specificity to the action of the RAD52 gene product. The effect of the RAD52 gene product on mitotic recombination is discussed later.

INTERMOLECULAR RECOMBINATION BETWEEN REPEATED SEQUENCES ON PLASMIDS

The development of a system that would allow the genetic detection of recombination between repeated sequences on autonomously replicating plasmids involved the transformation of a haploid cir+ can1 leu2 yeast strain with plasmid TLC-1, and analysis of the canavanine resistant recombination products.

Recombination between DNA sequences on plasmids can be shown by physical techniques. Electron microscopy and restriction enzyme cleavage established that Scp1 exhibited both intramolecular and intermolecular recombination (Guerineau et al., 1976). Subsequent work extended these

findings to recombination between Scp1 and artificially constructed hybrid plasmids (Broach et al., 1979; Gerbaud et al. , 1979;.Broach and Hicks, 1980; McNeil et al., 1980; Thomas and James, 1980). In such cases, efficient recombination depended on the presence of at least one copy of the inverted repeat sequence of Scp1 on the hybrid plasmid. Because efficient intramolecular recombination between the inverted repeat sequences of Scp1 requires the FLP gene (Broach and Hicks, 1980; Broach et al., 1982) , it is likely that the intermolecular recombination events utilized this specialized recombination system.

The formation of recombinants between Scp1 and various hybrid plasmids provided conclusive evidence for the exchange of DNA sequences between autonomously replicating plasmids in S. cerevisiae, but these recombinants could not be identified genetically. However, recombination between TLC-1 and Scp1 does generate phenotypically identifiable recombination products, and so provides the opportunity to analyze recombination between autonomously replicating plasmids in haploid S. cerevisiae.

Replicational Instability of Plasmid TLC-1

Plasmid TLC-1 contains the yeast CAN1 and LEU2 genes Therefore, can1 leu2 strains transformed with TLC-1 should be phenotypically CAN1 LEU2 . However, because of the replicative instability of TLC-1, cultures plated on medium containing canavanine and leucine appear canavanine resistant, so independent detection of the CAN1 marker is impossible. Because the ability to define the original genotype of the parents is essential to the definition of recombinants, this instability was investigated further.

When cultures of can1 leu2 strains containing TLC-1 are grown in liquid SC-leucine medium, about 30% of the cells in the culture cannot form colonies on SC-leucine plates because they do not contain sufficient copies of the LEU2 gene to support growth in the absence of leucine. When these cultures grown in liquid SC-leucine medium are plated on SC+canavanine plates, approximately 50% of the cells in the culture are capable of forming colonies. Therefore, when tested simply for the CAN1 marker, the can1 leu2 strains containing TLC-1 behave as though they were can1. However, only about 1 in 1,000 of the can1 colonies are LEU2, so the formation of the can1 colonies results from the selection of cells that have lost TLC-1. Because both failure to grow in the absence of leucine and ability to grow in the presence of canavanine are the result of loss of TLC-1, it appears that 20% of the cells in the culture have dual

characteristics. These cells appear to contain TLC-1 when plated on SC-leucine, but to lack TLC-1 when plated on SC-canavanine. This would occur if cells containing TLC-1 frequently budded off cells which did not contain TLC-1. If a round of cell division could be completed after a cell containing TLC-1 was plated on SC+canavanine, the bud may lack TLC-1 and be capable of forming a colony. If the same cell were plated on SC-leucine, the bud lacking TLC-1 would not be able to grow, but the mother cell would be capable of further budding and of forming a colony.

A number of factors influence the replicational instability of hybrid plasmids such as TLC-1, and therefore the frequency at which cells lacking the hybrid plasmid are formed. Yeast strains that carry the rad52-1 mutation lose plasmid TLC-1 from about 70% of the cells of an overnight culture grown in liquid YEPD; RAD52 strains lose the plasmid from only 50% of the cells under identical conditions. This suggests that rad52-1 has an affect on the stability of hybrid plasmids, but that the wild type gene product is not absolutely central to the maintenance of TLC-1. Recent evidence suggests that the RAD52 gene product may be involved in DNA repair synthesis (Orr-Weaver et al., 1981). It is possible that it is also involved in DNA synthesis associated with plasmid replication or segregation.

A second factor that influences the replicational stability of hybrid plasmid TLC-1 is the presence or absence

of the endogenous yeast plasmid Scp1. Yeast strains which are cir° do not stably maintain TLC-1. Growth of cir° strains containing TLC-1 in SC-leucine medium results in 5% of the cells in the culture remaining LEU2. This pronounced instability is because TLC-1 contains the Scp1 origin of replication, but lacks the Scp1 sequences required for efficient replication of this origin (Broach and Hicks, 1980). These sequences may code for trans acting proteins whose function is to override the normal replication cycle control of Scp1 (Zakian et al., 1979) to allow specific overreplication of the plasmid (Broach, 1982). Cells transformed with TLC-1 that lack Scp1 will contain only the initial number of plasmids obtained during the transformation, and this number will be maintained through subsequent rounds of cell division. Transformants containing Scp1 will be able to amplify the number of TLC-1 molecules, and therefore enhance the plasmid's stability.

The structure of the hybrid plasmid is also important in determining the replicational stability of the plasmid. Strains that carry the LEU2 sequence from TLC-1 inserted into the PstI site of Scp1 are more stable for the LEU2 phenotype than are strains which carry TLC-1 and Scp1. This relative difference in stability is seen both in RAD52 and rad52-1 strains. Other workers have noted that recombinant plasmids which contain only yeast DNA sequences are more stable than are plasmids which contain bacterial replicons

(Toh-e et al., 1980; Dobson et al., 1980b). How this detrimental effect is generated is not known.

Recombinational Instability of TLC-1

When can1 leu2 strains carrying TLC-1 are analyzed on plates lacking leucine, phenotypic changes that do not involve loss of the plasmid can be detected. When cultures of can1 leu2 cir+ strains carrying TLC-1 are plated on SC-leucine+canavanine plates, about 1 in 1,000 cells are capable of forming a colony. This frequency of can1 LEU2 cells is higher than would be expected for mutation of the CAN1 gene of TLC-1 to can1. Mutation of the chromosomal CAN1 gene in haploid S. cerevisiae occurs at a frequency of about 5×10^{-7}. Because the CAN1 gene in strains carrying TLC-1 is on a multicopy plasmid, and because CAN1 is dominant over can1, it might be expected that the formation of a can1 LEU2 derivative of a strain carrying TLC-1 would be a rare event. The observation that the frequency of can1 LEU2 cells is more than three orders of magnitude greater than for mutation of a single chromosomal CAN1 gene suggests either that mutation rates of genes on plasmids are much higher than those on the chromosome, or that some process other than mutation is generating the can1 LEU2 cells.

One way of distinguishing these two possibilities is through the rad52-1 mutation. Introduction of the rad52-1 mutation into can1 leu2 cir+ strains that carry TLC-1

reduces the frequency of can1 LEU2 cells from 10^{-3} to 10^{-5} per cell. Strains carrying the rad52-1 mutation have increased spontaneous mutation rates ; mutations at the chromosomal CAN1 gene are enhanced more than twenty-fold (from 5×10^{-7} to 130×10^{-7}) in rad52-1 strains. Because introduction of this mutator allele into can1 leu2 strains carrying TLC-1 results in a decrease in the frequency of can1 LEU2 cells, it is unlikely that the can1 LEU2 cells arise by mutation. On the other hand, the rad52-1 allele is known to reduce both meiotic (Prakash et al. 1980; Game et al., 1980) and mitotic (Prakash et al., 1980; Malone and Esposito, 1980) recombination. The fact that introduction of the rad52-1 mutation reduces the frequency of formation of can1 LEU2 cells suggests that these cells arise through some recombinational process.

Nature of the Recombination Process

A number of recombinational events could lead to the formation of can1 LEU2 derivatives of can1 leu2 strains containing plasmid TLC-1. Recombination between TLC-1 and the chromosome could, by a double cross-over or a gene conversion event, replace the CAN1 gene of TLC-1 with the can1 allele found on the chromosome. Subsequent selection for the can1 LEU2 phenotype would identify those cells whose entire complement of TLC-1 molecules carried the mutant can1 allele. Alternatively, recombination between TLC-1 and the

chromosome could replace, either by a double cross-over or a gene conversion, the chromosomal leu2 double mutant with the LEU2 gene of TLC-1. Subsequent selection for the can1 LEU2 phenotype would identify those cells which had a chromosomal LEU2 gene and had lost TLC-1. A third possibility involves recombination between TLC-1 and Scp1. Because the LEU2 region of TLC-1 is flanked by sequences from Scp1, a double cross-over or a gene conversion event could insert the LEU2 marker of TLC-1 into Scp1. Subsequent selection for the can1 LEU2 phenotype would identify those cells whose LEU2 plasmid population consisted entirely of the LEU2-Scp1 hybrid.

These models make different predictions about the structure of the DNA sequence in the canavanine resistant cells that contain the LEU2 marker. The first model predicts that the LEU2 marker is carried on a plasmid structurally identical to TLC-1. The second model predicts that the LEU2 marker is carried on the chromosome. The third model predicts that the LEU2 marker is carried on a plasmid structurally distinct from TLC-1. All lines of experimental evidence support the third model as being the major source of can1 LEU2 derivatives of can1 leu2 RAD52 cir+ strains carrying TLC-1.

The LEU2 marker in the can1 LEU2 derivatives of the can1 leu2 RAD52 cir+ strain transformed with TLC-1 is mitotically unstable. This eliminates the second model which predicts a mitotically stable chromosomal location for the

LEU2 marker. The LEU2 marker in the can1 LEU2 derivatives is, however, more mitotically stable than the LEU2 marker in strains carrying TLC-1. This suggests that there is a difference between TLC-1 and the LEU2 plasmid found in the can1 LEU2 derivatives.

Various tests confirm that the LEU2 plasmids in the can1 LEU2 strains are different from plasmid TLC-1. Southern analysis and transformation of E. coli with yeast DNA both show the presence of pBR322 sequences in yeast strains containing TLC-1, but both approaches fail to detect pBR322 sequences in the can1 LEU2 strains. Southern analysis also shows that although both the can1 LEU2 derivaties and the strains carrying TLC-1 contain a LEU2 plasmid, the plasmid in the can1 LEU2 strains is smaller than TLC-1.

Experiments also confirm that the LEU2 plasmid found in the can1 LEU2 derivatives of can1 leu2 cir+ strains containing TLC-1 had the structure expected for a recombinant between Scp1 and the LEU2 sequence of TLC-1. The plasmid is of the size expected for the Scp1-LEU2 hybrid, and it contains both the LEU2 sequence and regions of Scp1 that are not found in TLC-1. Because the LEU2 plasmid contains sequences not found in TLC-1, it could not have arisen through an intra-molecular rearrangement of TLC-1. Because these sequences came from the endogenous yeast plasmid, the LEU2 plasmid in the can1 LEU2 derivatives must have arisen by recombination between TLC-1 and Scp1.

Further experiments establish that the formation of the unstable LEU2 plasmid in the can1 LEU2 derivatives of the can1 leu2 strains containing TLC-1 requires the endogenous yeast plasmid. When strains containing TLC-1 are cir+, the frequency of can1 LEU2 derivatives is about 1×10^{-3} per cell; when the strains are cir°, the frequency of can1 LEU2 derivatives drops to about 2×10^{-5} per cell. Three can1 LEU2 derivatives from the cir° strain were analyzed; all had a mitotically stable LEU2 marker. This suggests that these strains were formed by loss of TLC-1 from a cell that had replaced the defective leu2 marker on the chromosome with the LEU2 gene of TLC-1. If the frequency of plasmid/chromosome recombination is the same in cir° and cir+ strains, it is evident that the vast majority of the can1 LEU2 derivatives obtained in the cir+ strains must have arisen through recombination between Scp1 and TLC-1.

Two events are required to generate cells containing the LEU2-Scp1 plasmid: formation of the plasmid, and segregation of the plasmid to a cell lacking TLC-1. The rad52-1 mutation could reduce the formation of can1 LEU2 derivatives of can1 leu2 strains containing TLC-1 either by reducing recombination between TLC-1 and Scp1, or by stablizing the segregation of TLC-1 to daughter cells. However, introduction of the rad52-1 allele was found to reduce the segregational stability of TLC-1, so the effect of the rad52-1 allele must be to decrease recombination

between Scp1 and TLC-1.

Dobson et al., 1980a have detected a plasmid similar to the present LEU2-Scp1 plasmid. Their plasmid, designated pYX, was observed in strains originally transformed with pJDB219, whose structure allows for the same recombinational events as does TLC-1. The presence of pYX was often correlated with loss of Scp1. This same phenomenon was also associated with the present LEU2-Scp1 plasmids, because 3 of 5 can1 LEU2 derivatives analyzed had lost Scp1. Therefore, the LEU2-Scp1 plasmid, derived from recombination between Scp1 and TLC-1, can displace the endogenous Scp1 plasmid, and can be used to select cir° strains. It is possible to directly select canavanine resistant derivatives of TLC-1 transformants, and these derivatives generate a sub-population highly enriched for cir° cells. This eliminates the need for extensive outgrowth of cultures and for random screening of leu2 strains to identify the cir° cells.

Reciprocal vs non-reciprocal plasmid-plasmid recombination

The recombination event which generates the can1 LEU2 strains could be either reciprocal or non-reciprocal. If the event was non-reciprocal, one product would be Scp1 with an inserted LEU2 fragment, the other product would be either TLC-1 or Scp1 (see Figure 15). If the event were reciprocal, one product would be Scp1 with an inserted LEU2 fragment, the other would be TLC-1 missing the LEU2 fragment (see

Figure 15. Formation of the LEU2-Scp1 plasmid by conversion. The top drawing (A) shows plasmid TLC-1 and Scp1. The middle drawing (B) shows these plasmids aligned at their regions of homology. The region of TLC-1 homologous to the small EcoRI fragment of ScpI contains the LEU2 region, so a conversion which replaces the small EcoRI fragment of Scp1 with the homologous fragment from TLC-1 will generate the LEU2-Scp1 plasmid shown in the bottom drawing (C). Similarly, a conversion which replaces the pBR322 and CAN1 regions of TLC-1 with the large EcoRI fragment of Scp1 will form the LEU2-Scp1 plasmid shown in (C).

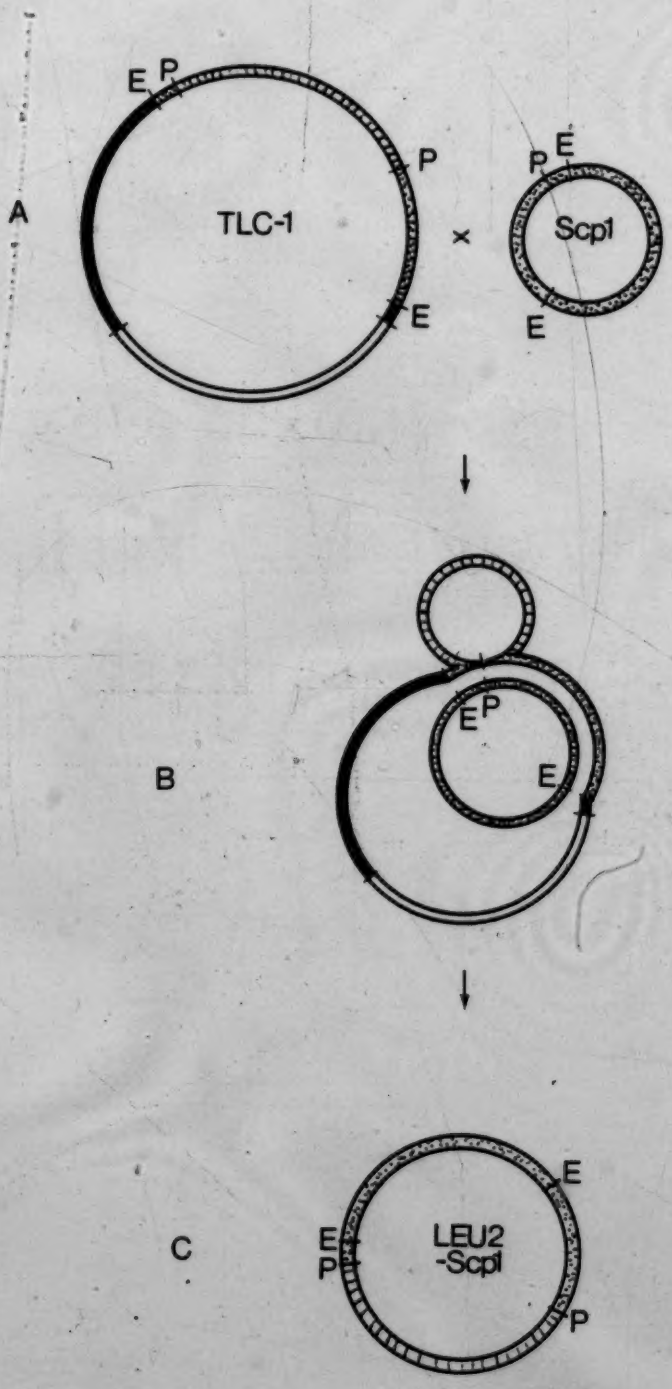
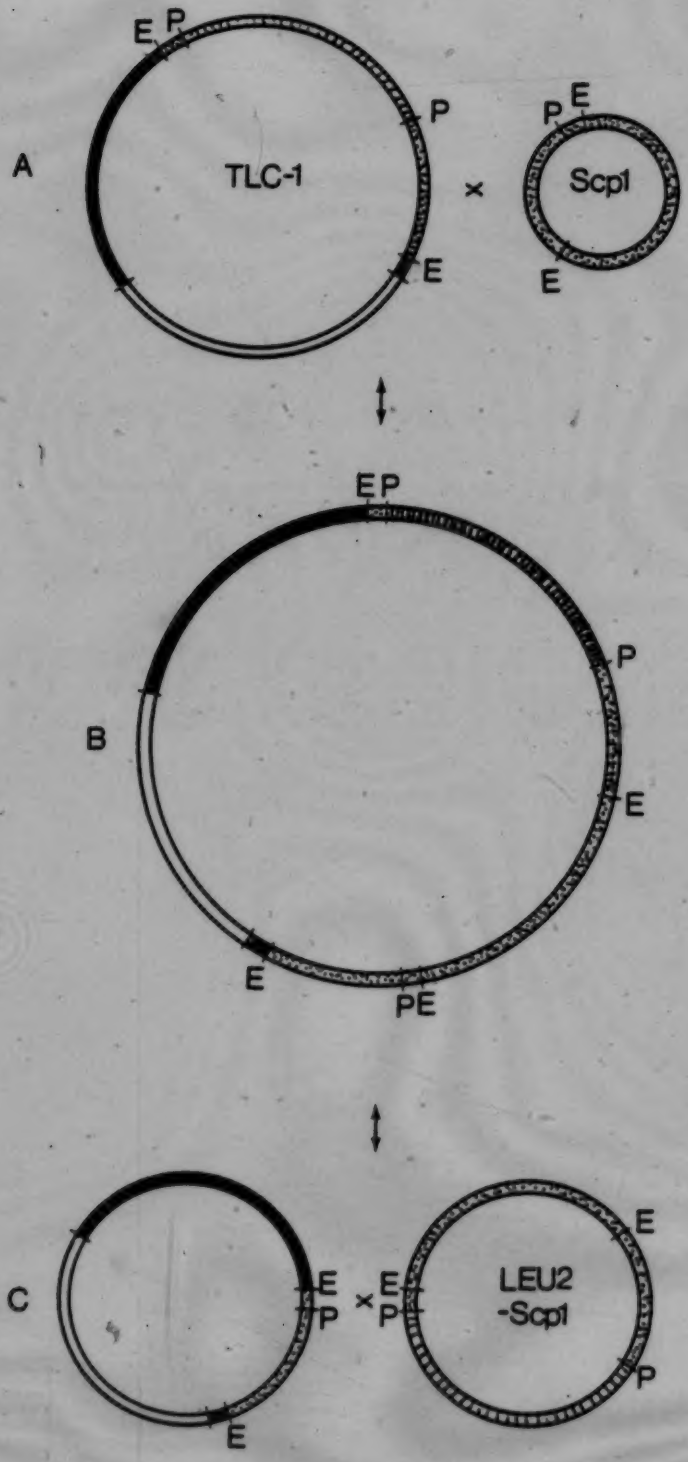

A
E
P
TLC-1
P
E
×
P
E
Scp1
E
B
E
P
E
C
LEU2
-Scp1
E
E
P
P

Figure 16). Only the LEU2-Scp1 recombination product can be genetically detected in this assay, and then only in strains that do not carry a functional CAN1 gene. Therefore, a distinction between reciprocal and non-reciprocal events cannot be made by direct analysis of the recombination products.

If the recombination process generating the LEU2-Scp1 plasmid was reciprocal, two crossovers would have to occur to exchange the LEU2 sequence from TLC-1 to Scp1. One crossover would have to occur either within the inverted repeat sequence itself, or within the 2.0 kb region, common to both plasmids, that includes co-ordinates 246 to 939 and 2407 to 3714 of the A form of Scp1, and encompasses one copy of the inverted repeat (Hartley and Donelson, 1980). The other crossover would have to occur within the 245 nucleotide region, common to both plasmids, that includes co-ordinates 0 to 245 of the A form of Scp1 (see Figure 16).

Crossing over between TLC-1 and Scp1 occurs frequently. Transformation of E. coli with DNA from yeast strains containing TLC-1 has detected larger molecules that appear to have arisen by reciprocal recombination between TLC-1 and Scp1 at their inverted repeat regions (Broach et al., 1979). This efficient recombination presumably is mediated by the site specific FLP recombination system (Broach et al., 1982). Reciprocal recombination between plasmids containing the 245 nucleotide homology has been detected during

Figure 16. Formation of the LEU2-Scp1 plasmid by reciprocal recombination. The top figure (A) shows plasmids TLC-1 and Scp1. A crossover between them at the large PstI-EcoRI fragment of both plasmids will form the integration product shown in figure (B). A further, intramolecular recombination between the directly repeated smaller PstI-EcoRI regions of homology will generate the two plasmids shown in the bottom drawing (C). One of these products is the LEU2-Scp1 plasmid.


A
TLC-1
Scp1
B
C
LEU2
-Scp1
E
P

transformation of cir+ strains with plasmid p47-3. Because this region does not contain DNA from the inverted repeat, this recombination event is likely to be mediated by generalized recombination systems of S. cerevisiae.

If the recombination process generating the LEU2-Scp1 plasmid was non-reciprocal, it would be necessary for the event to convert a large region of non-homology. Two different conversion events could form the LEU2-Scp1 plasmid. Plasmid TLC-1 carries the small EcoRI fragment of the B form of Scp1 with a 4.1 kb insertion of the chromosomal LEU2 region; conversion of the homologous region of the endogenous Scp1 plasmid into the sequence from TLC-1 will generate a LEU2-Scp1 plasmid. Plasmid TLC-1 lacks the large EcoRI fragment of the B form of Scp1, and carries, at the homologous position, an 8.85 kb sequence containing pBR322 and the chromosomal CAN1 gene; conversion of this 8.85 kb sequence into the large EcoRI fragment of the B form of Scp1 will also generate a LEU2-Scp1 plasmid. Both processes require the replacement of one sequence with another sequence that is homologous at the ends but contains large internal regions of non-homology (see Figure 15).

Conversion of extended regions of non-homology during meiosis have been detected genetically at the HIS4 (Fink and Styles, 1974; Fogel et al., 1978) and CYC1 (Lawrence et al., 1975) loci. Physical evidence for conversion of large regions of non-homology during mitosis have been provided

for the HIS3 gene (Scherer and Davis, 1980) and for the CAN1 duplication in the present study. In addition, mating type interconversion in haploid strains containing either the HO or ho allele appears to involve gene conversion of large regions of non-homology (Klar et al., 1980; Haber and Rogers, 1982). Therefore, both gene conversion and reciprocal recombination provide plausible mechanisms for the formation of the LEU2-Scp1 plasmid.

The observation that mitotic recombination between plasmids with Scp1 replicons requires the RAD52 gene product, which is also involved in recombination of chromosomal sequences, is consistent with evidence that although Scp1 is cytoplasmically inherited (Livingston, 1977), it has many chromosomal characteristics. Replication of Scp1 requires gene products also used for chromosomal replication (Petes and Williamson, 1975; Livingston and Kupfer, 1977), and synthesis of Scp1 DNA occurs at the same time as chromosomal replication (Zakian et al., 1979). The Scp1 molecule is also organized into nucleosomes (Livingston and Hahne, 1979; Nelson and Fangman, 1979), and although Scp1 is cytoplasmically inherited, its physical location appears chiefly nuclear (Kielland-Brandt et al., 1980).

It may be hoped that in addition to serving as a model system for studies of yeast chromosomal replication, Scp1 and derivatives of Scp1 may serve as a model system for investigations on mitotic chromosomal recombination. Because

cross-strand exchanges within the inverted repeats of Scp1 are detected during meiosis and not mitosis (Bell and Byers, 1979), it is possible that studies on Scp1 and Scp1-derived plasmids may be useful in understanding some aspects of meiotic recombination as well. Studies of prokaryotic recombination, both *in vivo* and *in vitro*, have been greatly facilitated by analysis of generalized recombination involving well defined small DNA molecules such as phage and plasmids (Potter and Dressler, 1978; Stahl et al., 1980).

Effect of *rad52-1*

Although the frequency of formation of *can1 LEU2* derivatives is greatly reduced in the presence of the *rad52-1* mutation, such derivatives do arise. Five *can1 LEU2* derivatives of MSW152-1A[TLC-1] were analyzed and classified into two groups. Three of the strains contained plasmids that were physically indistinguishable from the original plasmid TLC-1. These had replicational instabilities similar to TLC-1 during vegetative growth in yeast, they contained pBR322 sequences that were detectable by Southern blotting and transformation of *E. coli*, and the size of the plasmids in both yeast and *E. coli* was similar to TLC-1. The other two derivatives contained plasmids that appeared identical to the *LEU2*-Scp1 plasmids obtained from the *can1 LEU2* derivatives of *RAD52* strains. These plasmids were more replicationally stable in vegetatively growing yeast cells

than was TLC-1, they did not contain pBR322 sequences that could be detected by Southern blotting or E. coli transformation, and they were smaller than TLC-1.

The presence of LEU2-Scp1 plasmids in strains carrying the rad52-1 allele shows that the formation of these plasmids is not completely prevented by the rad52-1 mutation. Researchers have also noted that although the rad52-1 mutation can greatly reduce some forms of mitotic recombination, such as the integration of linear and gapped linear plasmids (Orr-Weaver et al., 1981) and gene conversion between his4 heteroalleles (Jackson and Fink, 1981), it never completely abolishes such events. This suggests that either the rad52-1 allele is slightly leaky and allows some residual recombinational activity, or that less efficient, RAD52 independent recombination pathways exist within the yeast cell.

The experiments on the duplicated his4 heteroalleles established that although the RAD52 gene product was necessary for efficient intrachromosomal mitotic gene conversion, it was not required for intrachromosomal mitotic reciprocal recombination (Jackson and Fink, 1981). As previously noted, the LEU2-Scp1 plasmid can be formed by reciprocal recombination, so it is possible that the low frequency of LEU2-Scp1 plasmids obtained in the rad52-1 strain is the result of RAD52 independent reciprocal recombination. This would suggest that virtually all the

LEU2-Scp1 plasmids in the RAD52 strains arise by non-reciprocal events that convert large regions of non-homology.

However, some apparent cases of reciprocal recombination, such as chromosomal integration of linear and gapped linear plasmids and resolution of certain direct repeats at the CAN1 locus, require the RAD52 gene product. Therefore RAD52 dependence may not be considered a definitive criterion for distinguishing between reciprocal and non-reciprocal recombination. Because the reciprocal events necessary to form the LEU2-Scp1 plasmid (FLP mediated recombination between Scp1 and TLC-1, and intramolecular recombination between 245 nucleotide direct repeats) appear to be very efficient, it seems unlikely that the LEU2-Scp1 plasmids would be formed at such low frequencies in rad52-1 strains if both these events were RAD52 independent. Therefore, the formation of the LEU2-Scp1 plasmid may be another example of a RAD52 dependent event that involves reciprocal recombination.

Three of the five can1 LEU2 derivatives obtained from the rad52-1 strain contained TLC-1 plasmids with a mutant allele of the CAN1 locus. These plasmids could have arisen either by mutation of the CAN1 gene, or by conversion between the plasmid and the chromosome which replaced the wild-type gene on the plasmid with the chromosomal can1 allele. The rad52-1 allele increases the frequency of

chromosomal can1 mutations by 20-fold, and reduces spontaneous mitotic gene conversion. Therefore, it is probable that the TLC-1 plasmids containing a mutant CAN1 allele were formed by mutation. Such plasmids provide readily obtainable defined sequences which could be used to identify the molecular consequences of rad52-1 specific mutagenesis.

CONCLUDING REMARKS

Mitotic recombination involving a variety of substrates has been investigated. It appears that three of the processes - intrachromosomal gene conversion between sequences which differ by a large region of non-homology, intrachromosomal resolution of an approximately 150 nucleotide non-contiguous direct repeat separated by 2.75 kb, and generalized recombination between autonomously replicating plasmids - involve the RAD52 gene product. Resolution of an approximately 300 nucleotide non-contiguous repeat separated by 8 kb did not involve the RAD52 gene product.

The observations that intramolecular gene conversion occurs between DNA sequences which differ by a 1.2 kb deletion, and that this process requires the RAD52 gene product, give strong support to suggestions that mating-type

switching occurs by gene conversion (Haber et al., 1980; Klar et al., 1980). Both intrachromosomal gene conversion and mating type switching involve interaction between sequences on the same chromosome (Strathern et al., 1980) and are reduced when the duplicated sequences are moved to separate chromosomes (Haber et al., 1981). Both processes also involve replacement of one sequence with another sequence containing extensive regions of non-homology (Strathern et al., 1980) and both require the RAD52 gene product (Malone and Esposito, 1980; Weiffenbach et al., 1981).

Recently, it has been noted that while the RAD52 gene product is necessary for processes such as intramolecular gene conversion (Jackson and Fink, 1981) and mating-type switching (Malone and Esposito, 1980; Weiffenbach et al., 1981), it is not required for events such as spontaneous unequal sister chromatid exchange (Zamb and Petes, 1981) and intrachromosomal reciprocal recombination (Jackson and Fink, 1981). In addition, the RAD52 gene product is required for the integration of linear and gapped-linear, but not circular non-replicative plasmids (Orr-Weaver et al., 1981) and is also required for UV induced sister chromatid exchange (Prakash and Taillon-Miller, 1981). Because DNA synthesis is potentially the common enzymatic process required for the RAD52 dependent events, it has been suggested that the RAD52 gene product is involved in repair

DNA synthesis (Orr-Weaver et al., 1981). The observation that the RAD52 gene product is required for resolution of some, but not all, non-contiguous direct repeats, and is also involved in generalized recombination between autonomously replicating plasmids, places constraints on this model.

Previous studies have attributed resolution of non-contiguous direct duplications to reciprocal recombination, either through unequal sister chromatid exchange or intramolecular looping out in a manner analogous to prophage excision (Jackson and Fink, 1981). Because these processes are RAD52 independent, the model predicts that they do not require DNA synthesis. However, resolution of the approximately 150 nucleotide direct repeats separated by 2.75 kb is RAD52 dependent, and so presumably does involve DNA synthesis. The efficient formation of the Scp1-LEU2 plasmid from recombination between Scp1 and TLC-1 is RAD52 dependent, and therefore involves DNA synthesis, although the plasmid could arise through RAD52 independent recombination processes analogous to the integration of circular plasmids into the chromosome (Orr-Weaver et al., 1981). Therefore, it is not possible to predict the RAD52 requirement on the basis of the structure of the recombining sequences.

It is evident that similar structures can have different requirements for the RAD52 gene product. Previous

studies (Jackson and Fink, 1981) have shown that reciprocal recombination between large repeats is not affected by the rad52-1 mutation. In the present study the RAD52 gene product is required for some, but not all reciprocal events. This suggests that there is an interaction between structure and enzymology during mitotic recombination. It is possible that this interaction is affected by the sequence of the recombining elements. Alternatively, the distance between the interacting DNA sequences may be the critical factor; chromosomal duplications separated by 2.75 kb may recombine by a different mechanism than those separated by 8 kb. The discriminatory capacity of the RAD52 gene product is not predicted by the recent model of the cellular activity of the RAD52 gene product (Orr-Weaver, et al., 1981). Because of the central importance of the RAD52 gene product in yeast recombination, determining the basis for this discrimination will be an important step toward understanding mitotic recombination in S. cerevisiae. One approach to investigating this process would be to develop recombinational substrates in which one of the two parameters of duplication size or distance between duplications is varied while the other is held constant. Another approach would be to hold both these parameters constant while varying the sequence of the recombining regions. This should define more clearly the discriminatory capacity of the RAD52 gene.

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APPENDIX 1
TLC-1

Plasmid TLC-1 (Broach et al., 1979) was treated with a variety of restriction enzymes to confirm the described structure. The co-ordinates identified in figure A1 were determined by comparison of the observed migration distance of the restriction fragments with molecular weight standards generated by HindIII and EcoRI plus HindIII digestion of lambda cI857 DNA (Davis et al., 1980).

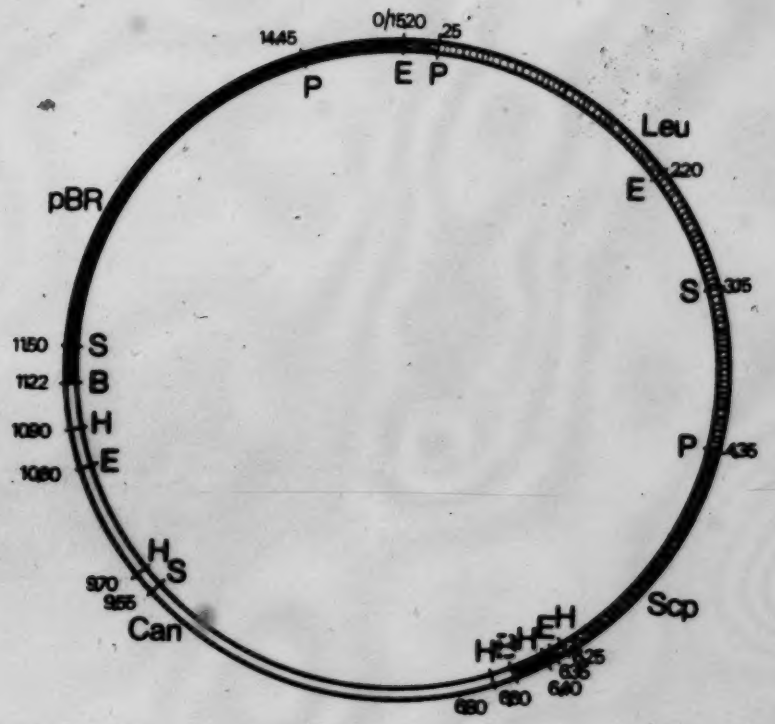
The regions of TLC-1 derived from pBR322 (Sutcliffe,1978) and Scp1 (Hartley and Donelson, 1980) have been sequenced; fragments from within these regions serve as internal checks of the estimated fragment sizes. The sizes determined from co-ordinates on Figure A1 agree well with the actual sizes of the sequenced regions. A comparison of the expected fragment sizes determined from the co-ordinates and the observed sizes determined from the gel shows the observed and expected size values agree within 10%.

The map determined in this study agrees well with the published map of TLC-1 (Broach et al., 1979). A HindIII site is included at position 6250 which is found in YEp13 (Broach et al., 1979), but is not placed on the map of TLC-1. However, the fragment of 3.2 kb generated by double digests of SalI and HindIII was reproducibly smaller than the doublet of the same size generated by double digests of SalI and EcoRI . This suggests that this HindIII site of YEp13 is also found in TLC-1.

Figure A1. Restriction map of plasmid TLC-1

The line drawing shows the approximate coordinates in nucleotides for the cutting sites of restriction enzymes EcoRI (E), PstI (P), SalI (S), HindIII (H), and BamHI (B). The dotted B represents a BamHI site that was not reformed after the cloning of the CAN1 region. The sequences derived from pBR322 are black, the sequences from Scp1 are stipled, the sequences from the LEU2 region of S. cerevisiae are hatched and the sequences from the CAN1 region of S. cerevisiae are white. The scale is 1 cm = 0.5 kb.

The photograph shows an agarose gel that has resolved restriction fragments from digestion of TLC-1 and lambda. Lane 1 is lambda cut with HindIII. Lane 11 is lambda cut with HindIII and EcoRI. Lanes 2-6 are digests of TLC-1 with EcoRI, HindIII, SalI, PstI and BamHI respectively. Lanes 7-10 are double digests of SalI cleaved TLC-1 recut with BamHI, HindIII, EcoRI and PstI respectively. The numbers on the ordinate represent a molecular weight scale in kilobases.


0/1520
25
1445
P
E
P
Leu
220
E
S
335
P
435
pBR
1150
S
1122
B
1090
H
1080
E
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S
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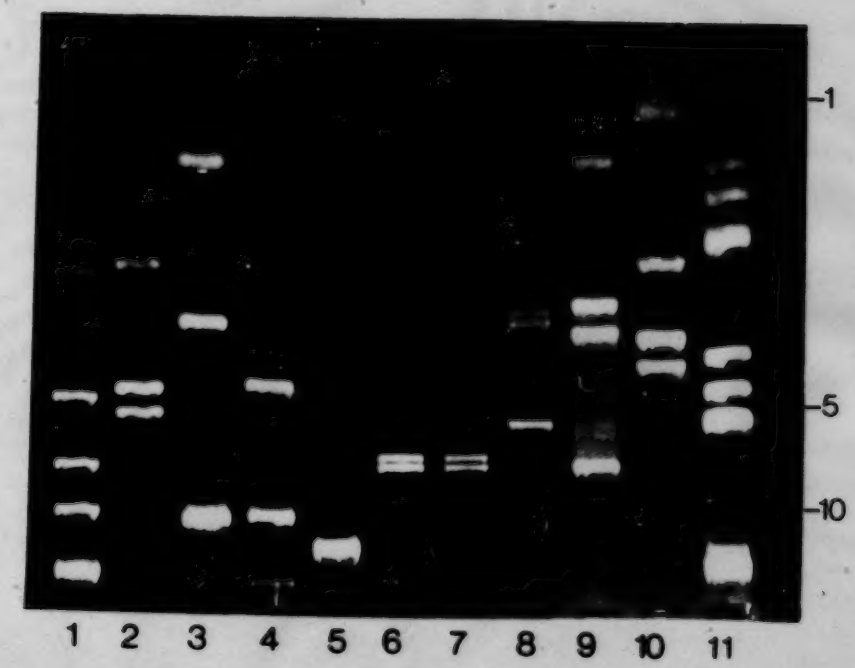
955
Can
Scp
680
690
640


1
5
10
1 2 3 4 5 6 7 8 9 10 11

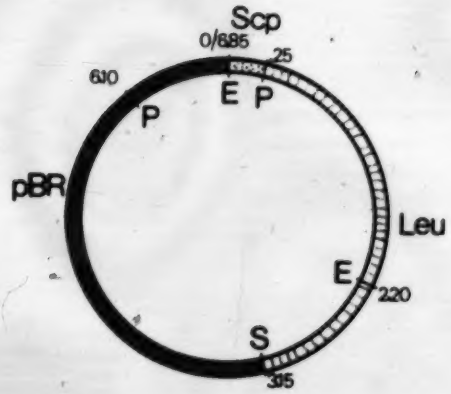
APPENDIX 2
p36

Plasmid p36 consists of a circular form of the 6.85 kb SalI fragment of TLC-1 that contains the pBR322 origin of replication and ampicillin resistance gene. The positions of the restriction sites within the 6.85 kb SalI fragment of TLC-1 are conserved in p36. The plasmid is not cleaved by HindIII or BamHI, is cleaved once by SalI , and is cleaved twice by both EcoRI and PstI .

Figure A2. Restriction map of plasmid p36

The line drawing shows the approximate coordinates in nucleotides for the cutting sites of restriction enzymes EcoRI (E), PstI (P), SalI (S), HindIII (H) and BamHI (B). The sequences of the plasmid derived from pBR322 are black, the sequences derived from Scp1 are stipled, the sequences derived from the LEU2 region of S. cerevisiae are hatched. The scale is 1 cm = 0.5 kb.

The photograph shows an agarose gel that has resolved the restriction fragments from digestions of plasmid p36 and lambda. Lane 1 is lambda cut with HindIII. Lane 7 is lambda cut with HindIII and EcoRI. Lanes 2-6 are digests of p36 with HindIII, EcoRI, SalI, BamHI and PstI respectively. The numbers on the ordinate represent a molecular weight scale in kilobases.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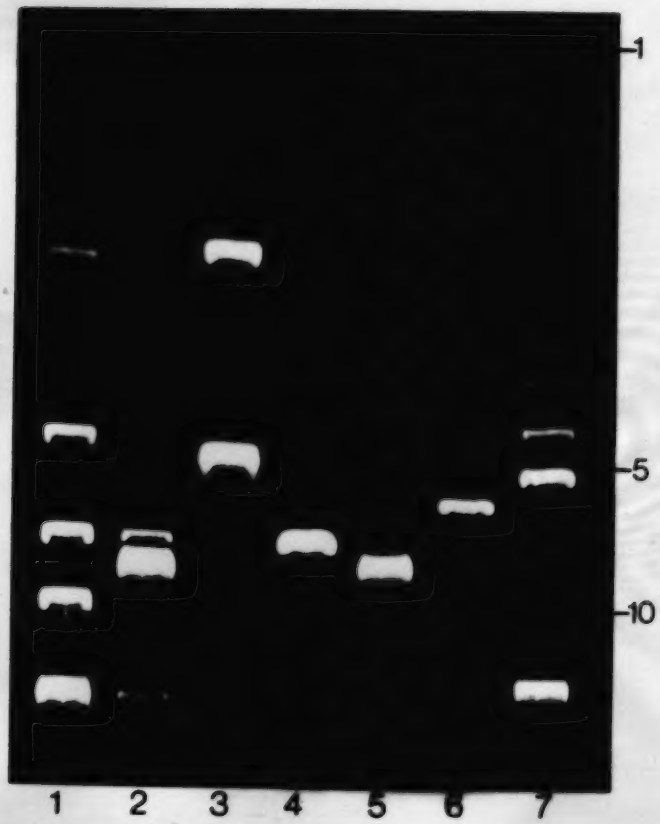
25
610
E P
P
pBR
Leu
E
220
S
395


1
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10
1 2 3 4 5 6 7

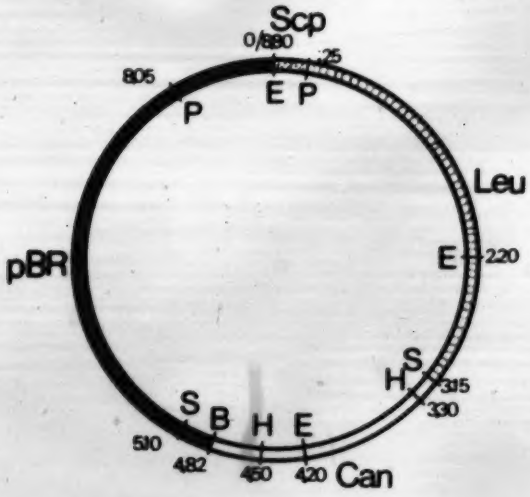
APPENDIX 3
p47

Plasmid p47 is a recombinant of the 6.85 kb and 1.95 kb SalI fragments of TLC-1. These two fragments could be arranged in the same orientation as they are found in TLC-1, or one fragment could be reversed in its orientation. No single restriction enzyme makes cuts within both fragments that are sufficiently asymmetric to orient the fragments. A double digest of p47 with both EcoRI and BamHI showed that the largest EcoRI fragment was cut with BamHI . This establishes that the orientation of the 6.85 and 1.95 kb fragments are the same in p47 as in TLC-1; if the orientation were reversed the smallest EcoRI fragment of p47 would have been cleaved by BamHI . The position of the restriction sites within the two SalI fragments of TLC-1 are conserved in p47.

Figure A3. Restriction map of plasmid p47.

The line drawing shows the approximate coordinates in nucleotides for the cutting sites of restriction enzymes EcoRI (E), PstI (P), SalI (S), HindIII (H) and BamHI (B). The sequences of the plasmid derived from pBR322 are black, the sequences derived from Scp1 are stipled, the sequences derived from the LEU2 region of S. cerevisiae are hatched, and the sequence derived from the CAN1 region of S. cerevisiae are white. The scale is 1 cm = 0.5 kb.

The photograph shows an agarose gel that has resolved the restriction fragments from digestions of plasmid p47 and lambda. Lane 1 is lambda cut with HindIII. Lane 7 is lambda cut with HindIII and EcoRI. Lanes 2-6 are digests of p47 with HindIII, EcoRI, SalI, BamHI and PstI respectively. The numbers on the ordinate represent a molecular weight scale in kilobases.


Scp
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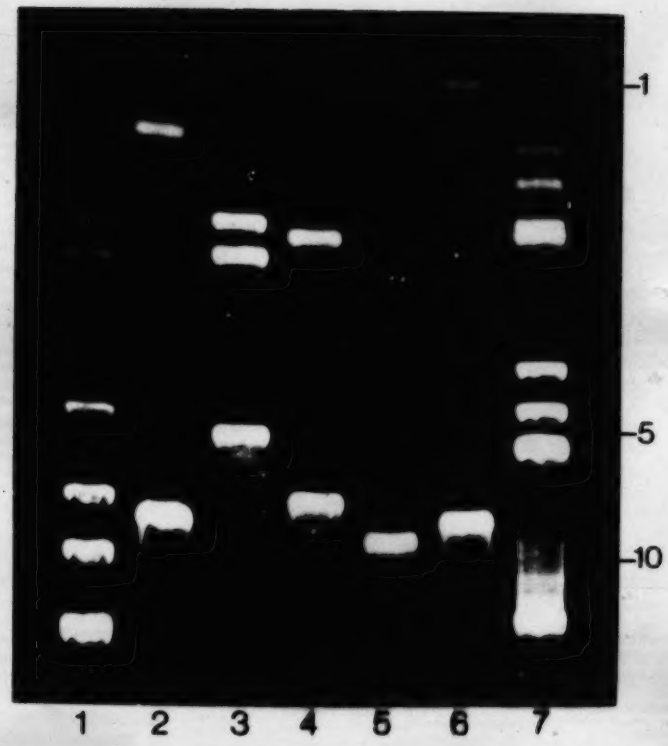
805
P
E P
Leu
pBR
E
220
S
H
315
330
S B H E
510
482
460
420
Can


1
5
10
1 2 3 4 5 6 7

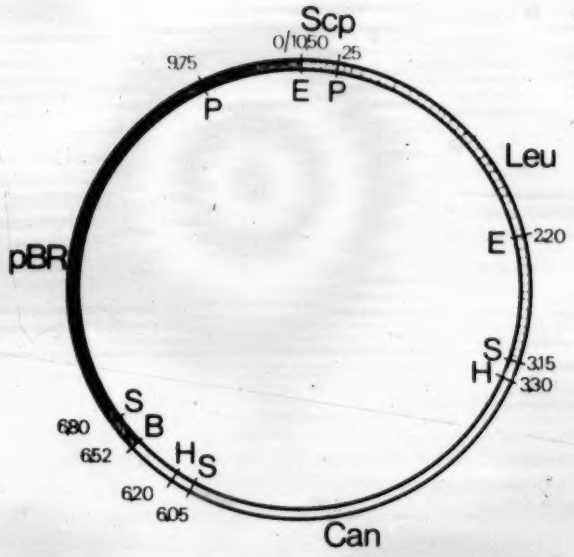
APPENDIX 4
p47-3

Plasmid p47-3 has replaced the 1.2 kb HindIII fragment of p47 with the 2.9 kb HindIII fragment of TLC-1. The 2.9 kb fragment could be inserted in one of two orientations relative to the 7.6 kb section of p47 containing the pBR322 origin of replication and amp gene. One orientation would repeat the 150 base pair sequence of co-ordinates 3.15 to 3.30 of p47, and co-ordinates 9.55 to 9.70 of TLC-1, as a direct repeat separated by 2.75 kb of the yeast CAN1 region; the other would place these sequences adjacent but reversed, generating a 300 nucleotide inverted repeat. Both the HindIII and SalI digestions of p47-3 generate fragments of 2.9 kb. This establishes that the orientation produces the non-tandem direct repeat of the 150 nucleotide sequence. The restriction sites within the 7.6 kb HindIII fragment of p47 and the 2.9 kb HindIII fragment of TLC-1 are conserved in p47-3.

Figure A4. Restriction Map of Plasmid p47-3.

The line drawing shows the approximate coordinates in nucleotides for the cutting sites of restriction enzymes EcoRI (E), PstI (P), SalI (S), HindIII (H) and BamHI (B). The sequences of the plasmid derived from pBR322 are in black, the sequences derived from Scp1 are stipled, the sequences derived from the LEU2 region of S. cerevisiae are hatched and the sequences derived from the CAN1 region of S. cerevisae are white. The scale is 1 cm = 0.5 kb.

The photograph shows an agarose gel that has resolved the restriction fragments from digestions of plasmid p47-3 and lambda. Lane 1 is lambda cut with HindIII Lane 7 is lambda cut with HindIII and EcoRI. Lanes 2-6 are digests of p47-3 with HindIII, EcoRI, SalI, BamHI, and PstI respectively. The numbers on the ordinate represent a molecular weight scale in kilobases.


Scp
0/1050
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975
P
E P
Leu
E
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S
H
315
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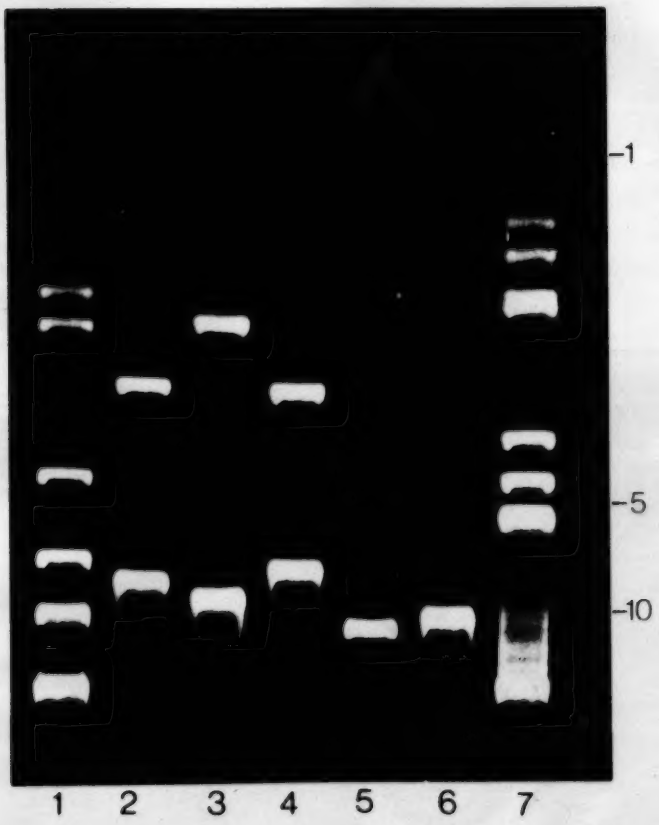
S
B
680
652
H
S
620
605
Can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APPENDIX 5
p47-3 AmpR #3

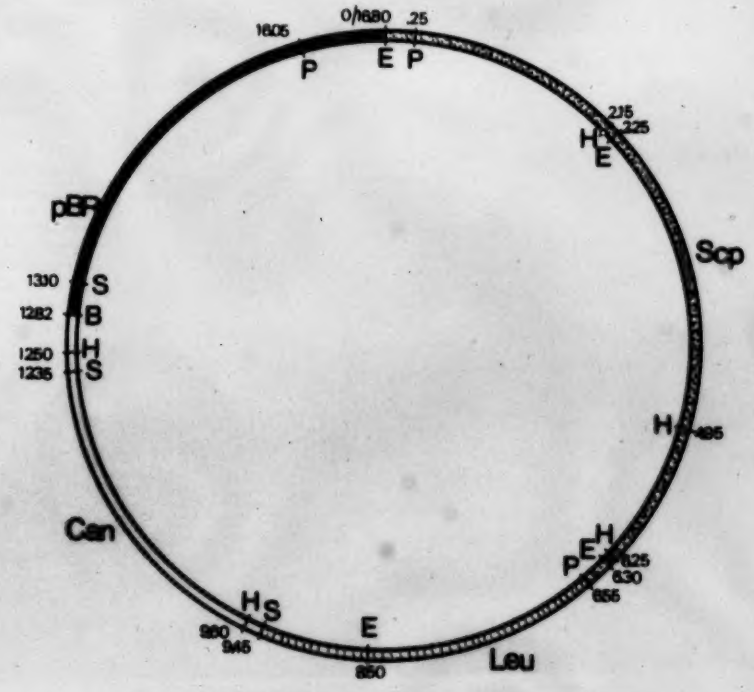
The ampicillin resistant E. coli transformants obtained from the DNA from yeast strain MSW5-21A/p47-3 #3 contain a plasmid larger that p47-3. This plasmid has approximately 6 kb more DNA then does p47-3, and contains three more HindIII cutting sites, two more EcoRI sites, and an additional PstI site. The extra DNA does not contain sites for the enzymes SalI or BamHI . The size of the DNA molecule and the number of restriction sites suggests that p47-3 has integrated into a copy of Scp1.

The most likely site of recombination between these two molecules is the 245 nucleotide sequence from co-ordinates 2407 to 2652 of the A form Scp1 (Hartley and Donelson, 1980) that is also found from co-ordinates 0 to 0.25 of plasmid p47-3. Because no HindIII fragment of approximately 0.9 kb was detected, it was likely that the Scp1 molecule is in the B configuration in the plasmid being studied. The restriction patterns obtained for p47-3 Amp R #3 are consistent with this plasmid being formed by a reciprocal recombination event between Scp1 and p47-3 within the 245 nucleotide region of homology.

Figure A5. Restriction map of plasmid p47-3Amp R #3.

The line drawing shows the approximate coordinates in nucleotides for the cutting sites of restriction enzymes EcoRI (E), PstI (P), SalI (S), HindIII (H) and BamHI (B). The sequences of the plasmid derived from pBR322 are black, the sequences derived from Scp1 are stipled, the sequences derived from the LEU2 region of S. cerevisae are hatched, and the sequences derived from the CAN1 region of S. cerevisiae are white. The scale is 1 cm = 0.5 kb.

The photograph shows an agarose gel that has resolved the restriction fragments from digestions of plasmid p47-3Amp R #3 and lambda. Lane 1 is lambda cut with HindIII. Lane 7 is lambda cut with HindIII and EcoRI. Lanes 2-6 are digests of p47-3Amp R #3 with EcoRI , HindIII, SalI, BamHI and PstI respectively. The numbers on the ordinate represent a molecular weight scale in kilobases.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P
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P
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225
H
E
Scp
pBR
1330
S
1282
B
1250
H
1235
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Can
P
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655
H
S
E
980
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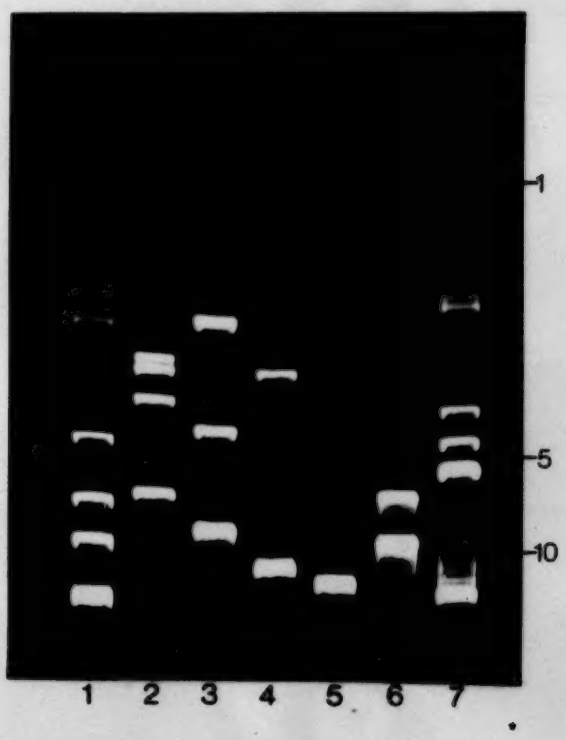
850
Leu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7

APPENDIX 6
pTM2

Plasmid Scp1 isolated from S. cerevisiae strain SS101 lacks the EcoRI site at position 2407 of the A form of the molecule (Livingston 1977). This plasmid exists within yeast cells in two forms (A and B) generated by recombination between the inverted repeats of the molecule. There are 4 arrangements possible for a hybrid of Scp1 and pBR322; either the A form or the B form of Scp1 could be joined to pBR322, and each form could be inserted in one of two orientations. Both pBR322 (Sutcliffe, 1978), and Scp1 (Hartley and Donelson, 1980) have been sequenced, so the structure of pTM2 was established by comparing the observed restriction pattern to the pattern expected for each arrangement.

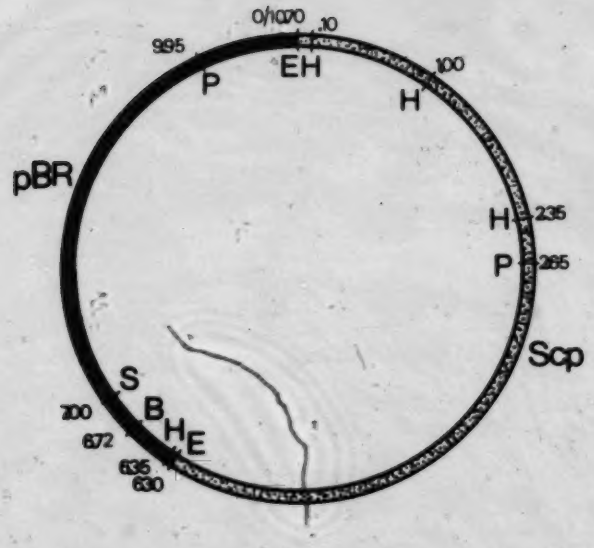
One possible arrangement would put the HindIII site at position 105 of both the A and B forms of Scp1 next to the HindIII site of pBR322. This would generate a HindIII fragment of 134 base pairs, and only three HindIII fragments would be found within the size range of the lambda molecular weight markers. This pattern was not observed; pTM2 produces four HindIII fragments of greater than 600 nucleotides.

In the other orientation, the A form of the plasmid would generate HindIII fragments of 4.438, 4.016, 1.314 and 0.912 kb, while the B form would generate HindIII fragments of 4.438, 2.711, 2.217, and 1.314 kb. The observed pattern of 4.75, 4.0, 1.25 and 0.92 kb is compatible with pTM2 containing the A form of Scp1. The remaining restriction enzyme digests are consistent with pTM2 containing the A form of Scp1.

Figure A6. Restriction Map of Plasmid pTM2.

The line drawing shows the approximate coordinates in nucleotides for the cutting sites of restriction enzymes EcoRI (E), PstI (P), SalI (S), HindIII (H) and BamHI (B). The sequences derived from pBR322 are black, and the sequences derived from Scp1 are stipled. The scale is 1 cm = 0.5 kb.

The photograph shows an agarose gel that has resolved the restriction fragments from digestion of lambda, Scp1, pBR322 and pTM2. Lane 1 is lambda cut with HindIII. Lane 9 is lambda cut with HindIII and EcoRI. Lanes 4-8 are digests of pTM2 with EcoRI, PstI, HindIII, SalI and BamHI respectively. Lane 2 is pBR322 cut with EcoRI, lane 3 is Scp1 cut with EcoRI. The numbers on the ordinate represent a molecular weight scale in kilobases.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Scp
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E
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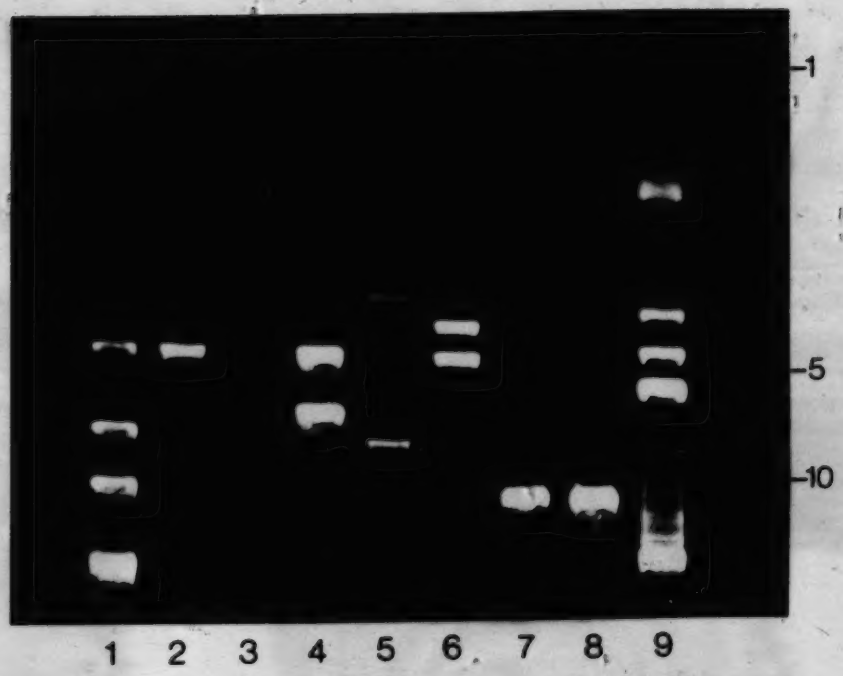
1
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10
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